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Characterization of Orphan CFTR Mutations

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Summary

Cystic Fibrosis (CF) is the most common lethal autosomal recessive disorder in the Caucasian population, affecting 1 in 2,500-6,000 newborns. CF is caused by mutations in the CF Transmembrane Conductance Regulator (*CFTR*) gene, being the most common a deletion of a phenylalanine residue at position 508 (F508del) that disrupts its traffic and function, due to protein misfolding. CFTR functions as a cyclic AMP-regulated chloride (Cl^-) and bicarbonate (HCO^{3-}) channel at the apical membrane of a variety of epithelial cells, also controlling several other ion channels and transporters, such as the epithelial sodium (Na^+) channel (ENaC). CFTR influences the ion and water content of the airway surface liquid (ASL). Its dysregulation, leads to a reduced ASL volume and consequently thick and dehydrated airway mucus. This thick mucus then impairs the mucociliary clearance (MCC) and causes the accumulation of bacteria and other pathogens, leading to persistent infections, inflammation and eventually to airway fibrosis and lung destruction, which is the primary cause of mortality in CF patients.

CFTR is synthesized at the endoplasmic reticulum (ER) where it is co-translationally core-glycosylated, generating an immature form (called band B). The protein is then processed during its trafficking through the Golgi apparatus to produce its fully-glycosylated mature form (band C) that functions as a channel at the cell surface.

About 2,000 alterations have been described to date in the *CFTR* gene, most presumed to be CF-causing. Ultimately, all of them result in defective cAMP-regulated Cl^- secretion by epithelial cells but due to different reasons. This great diversity led to the grouping of CFTR alterations into several classes according to the basic functional defect caused by each mutation, so as to be targeted by the same therapeutic strategy.

Among the ~2,000 CFTR mutations, many of them are very rare variants - termed “orphan” mutations due to their low frequency, >1,000 existing in less than 5 patients worldwide. For some of these mutations, the prediction of disease outcome is difficult, since the functional defect has not been characterized. In addition, as they affect very few patients, it is difficult not only to establish the diagnosis and prognosis, but also to assess the potential of new mutation-based therapies. Nevertheless, it is likely that some of these mutations will respond to already approved CFTR modulator drugs. Thus, there is an unmet need to functionally characterize these orphan mutations to establish validated drugs for mutation-based therapies.

The main goal of the present work was to better characterize a set of 11 CFTR orphan mutations which occur in Portuguese CF patients (P205S, L206W, R334W, R347P, I507del, R553P, R560S, L997F, H1079P, M1101K and D1152H), at the cellular level by novel cellular models and to test the efficacy of existing CFTR corrective drugs on these mutations. To accomplish this goal, the following tasks were proposed: (i) to generate novel cell lines stably expressing each of the CFTR mutants in study; (ii) to assess the molecular and functional effect of each CFTR mutant, using different approaches (Western blot, immunofluorescence and Ussing chamber); (iii) to test the efficacy of already approved drugs (for more common CFTR mutations) on these CFTR mutants expressed in the novel cell lines.

The first task was accomplished for the mutations P205S, R334W, R560S and H1079P. CF bronchial epithelial (CFBE) cell models stably expressing the above mutants were generated. The remaining mutations were studied using HEK293T cells transiently transfected with each CFTR mutant. All the above mutations were studied in terms of processing (no appearance of fully glycosylated form, i.e. band C in WB assays) and for both R560S and P205S, also in terms of function (Ussing chamber assays). For the mutations that showed processing defects, the corrector VX-809, part of the FDA/ EMA-approved lumacaftor/invacaftor drug, was tested.

Overall the results showed that:

1. Mutations P205S, L206W, I507del, R553P, R560S, H1079P and M1101K cause processing defects (abrogating or drastically reducing the production of band C).
2. Among these seven mutations affecting processing, P205S-, L206W-, R553P- and M1101K-CFTR are rescued by the corrector VX-809.
3. Mutations R334W, R347P, L997F and D1152H did not cause a defect in processing – thus are likely to cause CF by impairing CFTR function.

The continuation of the work developed during this project will be important to further characterize the cellular effect of each mutation and to assess whether they can be rescued by the already approved drugs in order to contribute to bringing these novel therapeutic approaches to CF patients carrying such rare mutations.

Keywords: CFTR, Cystic Fibrosis, Orphan mutations.

Resumo

A fibrose quística (FQ) é a doença autossómica recessiva letal mais comum na população caucasiana, afetando 1 em 2500-6000 recém-nascidos. Esta doença é causada por mutações no gene *Cystic Fibrosis Transmembrane Conductance Regulator* (*CFTR*). A proteína CFTR funciona como um canal de cloreto (Cl^-) e bicarbonato (HCO^{3-}) regulado por cAMP e é expressa na membrana apical de várias células epiteliais. Para além disso, a CFTR também regula outros canais de iões e transportadores, entre os quais o canal de sódio (Na^+) epitelial (ENaC). Desta forma, a CFTR influencia o conteúdo em água e iões do líquido que reveste as vias respiratórias (*airway surface liquid* - ASL). A sua desregulação leva a uma redução do volume do ASL e consequentemente a um muco muito espesso e desidratado que impede a limpeza mucociliar e leva à acumulação de bactérias e agentes patogénicos. Isto leva a infeções persistentes, inflamação e, eventualmente, à fibrose e destruição do tecido das vias respiratórias, sendo esta a principal causa de morte.

A proteína CFTR pertence à superfamília dos transportadores ABC (do inglês *ATP binding cassette*), sendo constituída por 5 domínios distintos: dois domínios transmembranares (MSDS1 e MSD2), formados por 6 segmentos transmembranares cada, e que em conjunto constituem o poro do canal; dois domínios de ligação ao ATP (NBD1 e NBD2) e um domínio regulador (R), que contém múltiplos locais de fosforilação – sendo responsáveis pela abertura e fecho do canal

A proteína CFTR é sintetizada no retículo endoplasmático onde é glicosilada co-traducionalmente, dando origem a uma forma imatura (designada band B). Esta proteína sofre depois processamento, durante o seu tráfego pelo complexo de Golgi, originando uma proteína madura (banda C) que vai para a membrana celular onde funciona como canal de cloreto.

Até à data, já foram descritas mais de 2000 mutações no gene *CFTR*, a maior parte das quais causadoras de fibrose quística. A deleção do resíduo de fenilalanina na posição 508 (F508del) é a mutação mais comum e causa um defeito no trafego e na função devido ao *misfolding* da proteína. Eventualmente, todas as mutações resultam num transporte deficiente de água e iões no epitélio. No entanto, de acordo com o defeito provocado por cada mutação, essa alteração no transporte pode ser mais ou menos grave (ausência total ou transporte residual de cloreto no epitélio, respetivamente). Assim, as mutações foram agrupadas em sete classes de acordo com o defeito causado. Nas mutações de classe I não há produção de proteína, sendo normalmente mutações *nonsense*, nas quais a existência de um codão stop prematuro leva à degradação do mRNA por *nonsense-mediated decay* (NMD); as mutações de classe II levam ao *folding* e processamento incorreto da CFTR, ficando a proteína retida no reticulo endoplasmático (RE) e posteriormente degradada pelo sistema ubiquitina-proteassoma; as mutações de classe III impedem o funcionamento do canal, sendo maioritariamente mutações localizadas nos NBDs; as mutações de classe IV levam a uma baixa condutância, localizando-se maioritariamente nos MSD1 e MSD2; as mutações de classe V resultam em diminuição dos níveis de CFTR, normalmente devido a defeitos no *splicing*; nas mutações de classe VI, a proteína é pouco estável na membrana celular; e por fim, as mutações de classe VII levam a que não haja produção de mRNA CFTR, sendo muito difícil a sua correção usando fármacos (ex: grandes deleções).

As consequências funcionais causadas por uma determinada mutação podem gerar defeitos celulares que resultam na inclusão de uma dada mutação em mais que uma classe - é o caso da mutação F508del que possui defeitos que tanto a classificam como uma mutação de classe II (retenção intracelular) como de classe III (abertura reduzida do canal).

De entre as cerca de 2.000 mutações no gene *CFTR*, muitas são variantes pouco comuns, sendo designadas de mutações "órfãs". Para estas mutações, é difícil prever os efeitos da mutação pois a mutação ainda não foi caracterizada em termos funcionais e porque na maioria dos casos existem em heterozigotia com outras mutações. Para além disto, como estas mutações afetam poucos pacientes, é

difícil não só fazer o diagnóstico, mas também testar os fármacos já existentes (e aprovados) para mutações mais comuns. Assim, há uma necessidade de fazer uma caracterização funcional destas mutações órfãs a fim de se poder fazer a validação de compostos que levem à correção especifica de cada mutação.

Para o estudo da fisiopatologia da fibrose quística têm sido desenvolvidos vários modelos. De forma a se poder estudar a CFTR no seu ambiente natural, ou seja, na membrana das células, é importante usar células com um fenótipo epitelial e que tenham capacidade de polarizar e estabelecer *tight junctions* para se poder medir o transporte iónico. Estas células são normalmente imortalizadas e são manipuladas de forma a que expressem a proteína CFTR *wt* ou mutante. Quando é requerido um modelo que represente melhor o epitélio das vias respiratórias, são usadas células primárias do epitélio nasal ou dos brônquios - estas podem ser obtidas através de biópsias/escovados ou pólipos nasais ou isolados a partir de materiais de doentes após transplante pulmonar. Outro tipo de sistema são os organoides intestinais, que são produzidos a partir de células estaminais primárias das criptas do reto e podem ser cultivados em matrigel por longos períodos de tempo sem modificações genéticas. Este sistema tem a vantagem de poder ser usado para ensaios funcionais nos quais a ativação da CFTR pela forskolin leva à secreção de sais e fluido para o lúmen no organoide, levando ao seu inchamento *(forskolin-induced swelling* - FIS).

Assim, embora seja fisiologicamente relevante o uso de materiais de doentes para estudar os mecanismos de doença e testar possíveis estratégias terapêuticas, estes necessitam de ser complementados com modelos celulares, pois a maioria das mutações aparecem em heterozigotia, e é difícil de perceber o efeito de cada uma individualmente. Assim as linhas celulares produzidas neste trabalho são indispensáveis para o estudo de mutações raras.

Este trabalho teve como principal objetivo a caracterização de mutações órfãs no gene *CFTR* - nomeadamente as mutações P205S, L206W, R334W, R347P, I507del, R553P, R560S, L997F, H1079P, M1101K e D1152H. Estas foram estudadas a nível funcional e molecular, usando novos modelos celulares. Para além disto, testámos se os fármacos já existentes para mutações comuns são eficazes nestas mutações. Para isto foram levadas a cabo as seguintes tarefas:

(i) Criação de novas linhas celulares (baseadas na linha CFBE) que sobreexpressam de forma estável cada uma das mutações em estudo;

(ii) Estudo do efeito de cada mutação a nível funcional e molecular usando diferentes técnicas (Western blot, imunofluorescência, câmara de Ussing);

(iii) Avaliação do efeito de drogas (já aprovadas para mutações comuns) nestas novas linhas celulares.

A primeira tarefa foi concluída para as mutações P205S, R334W, R560S e H1079P. Células CFBE (*Cystic fibrosis bronchial epithelial*) foram usadas como modelo celular, tendo sido estavelmente transduzidas com cada mutante (com recurso a vetores lentivirais). As restantes mutações foram estudadas usando células HEK293T transfetadas transientemente. Todas as mutações foram estudadas em termos de processamento (presença ou ausência da forma matura da proteína - banda C - por WB) da proteína CFTR e as mutações P205S e R560S foram também estudadas do ponto de vista funcional (determinação de transporte transepitelial em câmara de Ussing). Para as mutações em que se observou um defeito no processamento, foi testado o corrector VX-809 - componente do fármaco combinado lumacaftor/ivacaftor aprovado pela FDA e EMA.

Os resultados neste trabalho mostram que:

- As mutações P205S, L206W, I507del, R553P, R560S, H1079P e M1101K levam a um defeito no processamento (banda C ausente ou bastante reduzida).
- O defeito de processamento das mutações L206W, R553P e M1101K é corrigido pelo corretor VX-809.

- O defeito de processamento causado pelas mutações I507del e H1079P não é corrigido pelo VX-809.
- O defeito de processamento causado pela mutação R560S não é corrigido por nenhum dos compostos testados (VX-809, VX-661, cisteamina – isolada ou combinada com o epigalocatequinagalato) nem pela incubação a baixa temperatura.
- As mutações R334W, R347P, L997F e D1152H não levam a um defeito no processamento, gerando possivelmente uma deficiência a nível funcional.

A continuação do trabalho desenvolvido neste projeto será importante para a melhor compreensão do efeito de cada mutação e para a identificação de compostos já disponíveis (aprovados ou em ensaio clínico) levam à sua correção. Assim este trabalho contribuirá para a descoberta de novas abordagens terapêuticas para pacientes que possuem mutações raras como as que aqui foram estudadas.

Palavras-chave: CFTR, Fibrose quística, Mutações Orfãs.

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Abbreviations

ABC	ATP binding cassette
ALI	Air-liquid interface
APS	Ammonium persulfate
ASL	Airway surface liquid
ATP	Adenosine triphosphate
AUC	Area under the curve
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CF	Cystic Fibrosis
CFBE41o- / CFBE	Cystic fibrosis bronchial epithelial (cell line)
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
C-terminal	Carboxyl-terminal
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EGCG	Epigallocatechin gallate
ENaC	Epithelial sodium channel
EMEM	Eagle's minimum essential medium
ER	Endoplasmatic reticulum
ERQC	Endoplasmatic reticulum quality control
FBS	Fetal bovine serum
FIS	Forskolin induced swelling
FRT	Fisher rat thyroid
Fsk	Forskolin
hBE	Human bronchial epithelial cells
HEK 293 T	Human embryonic kidney (cell line)
hNEC	Human nasal epithelial cells
IBMX	3-isobutil-1-metilxantina
ICL	Intracellular loop
LB	Lysogeny broth
MI	Meconum ileus
MSDs	Membrane-spanning domains
NBD	Nucleotide-binding domains
NMD	Nonsense-mediated decay
N-terminal	Amino-terminal
PAGE	Polyacrilamine gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Pancreatic insufficiency
PM	Plasma membrane
PTC	Premature termination codon
PVDF	Polivinylidene difluoride
RD	Regulatory domain
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulfate
TEER	Transepithelial electrical resistance
TEMED	Tetramethylethylenediamine
TM	Transmembrane domain

$\mathbf{V_{te}}$	Transepithelial voltage
WB	Western Blot
wt	Wild type

1. Introduction

1.1. Cystic Fibrosis

Cystic fibrosis (CF) is the most common life-shortening autosomal recessive disease in the Caucasian population [1] affecting one in 2,500-6,000 new-borns and having a carrier frequency of 1 in 25 to 40 individuals, depending on the geographic region [2].

The first description was provided by Dorothy Anderson in 1938, that called the disease "cystic fibrosis of the pancreas" reflecting the destruction of the pancreas that is one of hallmarks of CF [3]. Although a defect in chloride (Cl^-) transport was identified as a characteristic of CF, it was only in 1989 that the gene responsible for CF was identified and named CF Transmembrane Conductance Regulator (CFTR) [4]. This gene encodes a cAMP-dependent Cl^- and bicarbonate (HCO_3^-) channel expressed at the apical plasma membrane (PM) of epithelial cells [4]. Mutations in the gene were found in all CF patients analysed [5], the most common of which is F508del, a three-nucleotide deletion causing the deletion of phenylalanine residue at position 508 leading to defective processing and trafficking of CFTR protein [6].

The basic defect in CF is associated with decreased Cl^- ion conductance across the apical membrane of epithelial cells [4]. Although CFTR functions mainly as a chloride channel, it is a major epithelial ion regulator, that includes inhibition of sodium (Na^+) transport through the epithelial Na^+ channel (ENaC) [7]. Lack of CFTR leads to a general dysregulation, that will cause dehydration of the airway surface liquid layer (ASL), resulting in enhanced mucus viscosity and in impaired mucociliary clearance (MCC) [8]. As MCC is an important defence mechanism against pathogens, its reduction in CF patients leads to chronic lung infections normally by *Pseudomonas aeruginosa* associated with inflammation and scarring resulting in progressive loss of lung function. This process is the so-called CF pathogenesis cascade [2] (**Figure 1.1**).

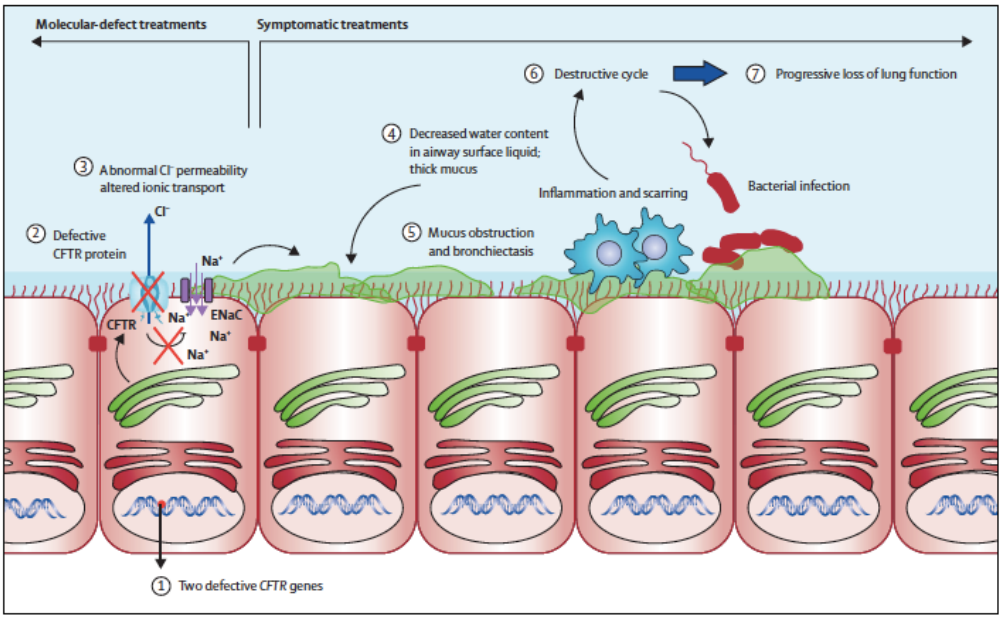


Figure 1.1. **Pathogenetic cascade that causes cystic fibrosis lung disease.** Cystic fibrosis is caused by mutations in the *CFTR* gene, which leads to a series of events and ultimately leads to loss of lung function [9].

Other CF symptoms include pancreatic dysfunction, elevated sweat Cl^- and male infertility, but the obstructive lung disease remains the leading cause of morbidity and is responsible for 80% of mortality [10], [11].The symptoms vary across CF patients and may change over the patient's life [10], [12], [13]. Exocrine pancreatic insufficiency (PI) is present in about 85% of CF patients. Pancreatic disease is believed to result from a reduced volume of pancreatic secretion with low concentrations of HCO_3^-. Without sufficient fluid and HCO_3^- digestive proenzymes are retained in pancreatic ducts being prematurely activated and leading to tissue destruction and fibrosis [14]. Moreover, the CF phenotype may include less common symptoms or complications such as meconium ileus (MI), distal intestinal obstruction syndrome, pancreatitis, liver disease or diabetes, among others [13]. Concerning the reproductive system, 98% of men are infertile and women are also frequently affected [14].

Since CF patients have elevated Cl^-concentrations in the sweat, the sweat test remains the most readily available and clinically useful way of making the diagnosis of CF [10]. A sweat chloride concentration above 60mmol/L on repeated analysis corresponds to a CF diagnosis, but about 5% of these cases are false negative. Genotyping the most common CFTR mutations is then used to confirm the diagnosis [14] – however, the diagnosis will be missed if the patient is affected by a mutation that is not screened [10].

Typically, the CF symptomatic treatments include mucolytics to dissolve the thick mucus, antibiotics to treat or prevent infections and anti-inflammatory agents to reduce chronic inflammation [9]. Because of all the progress in the study of this disease the median predicted survival age in 2015 for the USA was 41.6 years, having increased 8.2 years since 2013 [15].

Despite of these major therapeutic advances, the burden of CF care is still very high and life expectancy and quality of life of most CF patients are still limited [11]. The on-going study of the CFTR gene and protein has provided new opportunities to develop novel therapeutic approaches to the treatment of CF. These may include CFTR gene replacement, suppression of nonsense mutations, restoration of folding and function of mutant CFTR channels [16]. *In vivo* gene therapy trials in patients with CF have been done with viral vectors and cationic lipids, however long-term effects were limited, so it is presently not a treatment option [14]. Alternatively, the approaches with small molecules that aim to rescue intracellular production, trafficking or activation of CFTR channel appear to be the most attractive in a short- to medium-term period [11]. The available CFTR modulators are correctors and potentiators which are able to rescue F508del-CFTR protein to the PM and can restore gating of the mutant channel, respectively [16]. Nonetheless, there are still mutations that do not respond to these compounds, therefore, therapeutic approaches that include read-through of premature termination codons (PTCs), nucleic acid approaches or take advantage of other ion channels and transporters to compensate the CFTR absence are necessary for the treatment of CF patients [17].

1.2. CFTR gene and protein

1.2.1. CFTR gene

The *CFTR* gene was identified by positional cloning techniques and located on the long arm of chromosome 7 (7q31). It consists of 27 exons and encodes a 1,480 amino-acid protein. Analysis of 3.8 kb of genomic sequence upstream of exon 1 of this gene revealed a high GC content (65%), no TATA box and multiple transcriptional start sites [18].

More than 2,000 alterations have so far been described, including missense (39.6%), frameshift (15.6%), splicing (11.4%), nonsense (8.3%), large in-frame deletions or insertions (2.6%, 2.0%, respectively), promoter (0.7%) mutations and non-pathological variants (15%)[9], [19]. All these mutations have been reported in the CFTR1 database [19], with the clinical features available for the most common variants in the *CFTR2* database [20].

1.2.2. CFTR – Protein structure and function

CFTR is a symmetrical, polytopic protein that belongs to the superfamily of the ATP-binding cassette (ABC) transporters, which bind ATP and use the energy to drive the transport of a wide variety of substrates across extra- and intracellular membranes [21]. CFTR, in particular, has adapted the ABC structural motif to form a tightly regulated anion channel at the apical surface of many epithelia [22].

CFTR is composed by five domains (**Figure 1.2**): two membrane-spanning domains (MSDs) each containing six alpha-helical segments, that form the channel pore; two nucleotide-binding domains (NBD1/2) that interact with ATP to control channel gating; and a central unique regulatory domain (RD), absent in other ABC transporters and containing multiple consensus phosphorylation sites [23], [24]. Both the amino (N) and carboxyl (C) terminal tails of CFTR are in the cytoplasm and mediate the interactions of CFTR with a variety of binding proteins [21]. When ATP binds to CFTR, NBDs heterodimerize and hydrolysis of one of the ATPs disrupts the NBD1-NBD2 interaction and closes the channel gate, leading to the termination of the anion flow.

Like other proteins from the secretory pathway, CFTR assembly begins with the synthesis and folding in the endoplasmic reticulum (ER), where it is core-glycosylated. Once checked for correct folding by the ER quality control (ERQC), this immature form of CFTR migrates to the Golgi complex, where it undergoes processing to achieve its mature form [23].

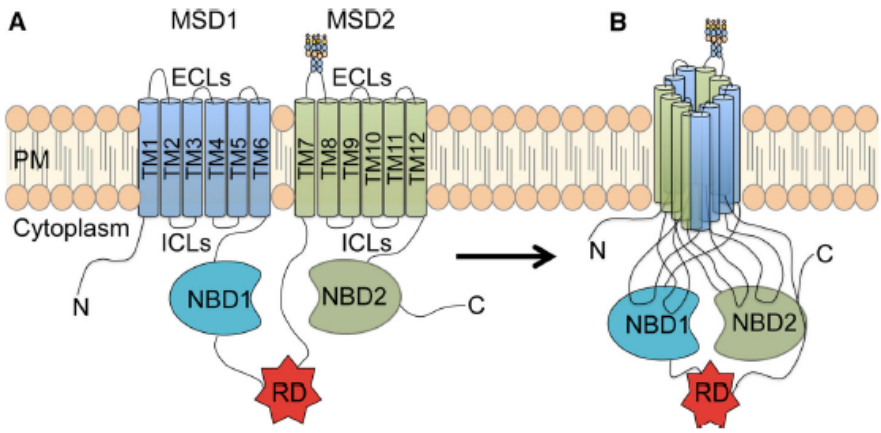
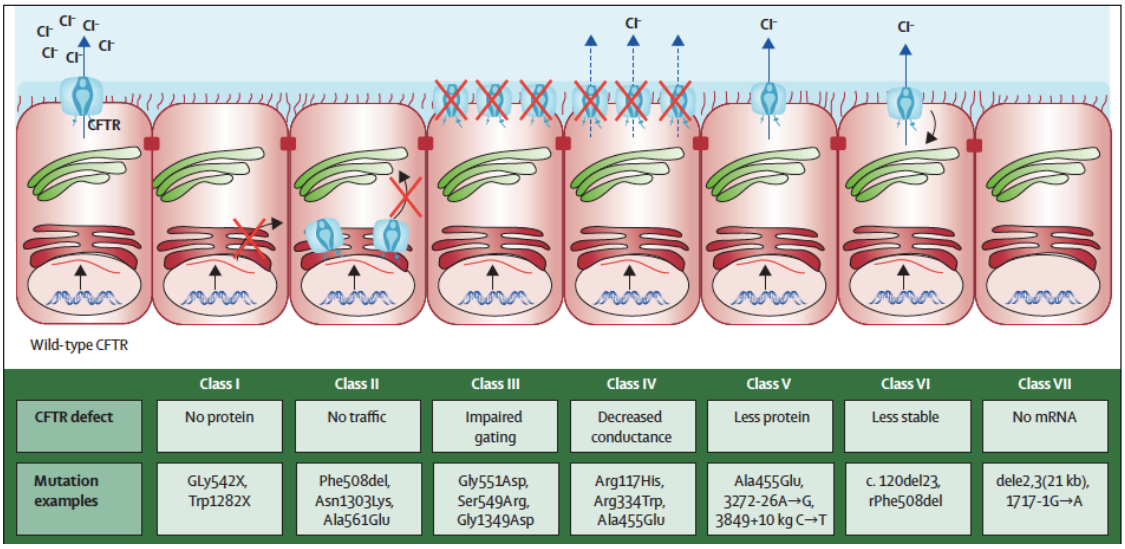


Figure 1.2. **CFTR protein structure.** It is composed of five domains: two membrane-spanning domains (MSD1 and MSD2), each one composed of six transmembrane segments (TM1-6 and 7-12), two cytosolic nucleotide binding domains (NBD1 and NBD1), and a regulatory domain (RD)[24].

1.2.3. CFTR mutation classes

Mutations in CFTR result in abnormal epithelial ion and water transport, and according to the defect in CFTR they may be "severe" (leading to total absence of Cl^- transport through CFTR) or "mild" (residual Cl^- transport through CFTR)[25], [26]. CFTR mutations have been classified according to their functional defect into seven classes (**Figure 1.3**).



	Class I	Class II	Class III	Class IV	Class V	Class VI	Class VII
CFTR defect	No protein	No traffic	Impaired gating	Decreased conductance	Less protein	Less stable	No mRNA
Mutation examples	GLy542X, Trp1282X	Phe508del, Asn1303Lys, Ala561Glu	Gly551Asp, Ser549Arg, Gly1349Asp	Arg117His, Arg334Trp, Ala455Glu	Ala455Glu, 3272-26A→G, 3849+10 kg C→T	c. 120del23, rPhe508del	dele2,3(21 kb), 1717-1G→A

Figure 1.3. **Classes of CFTR mutations**. Mutations are grouped into seven functional classes [9]. Examples of the most common mutations in each class are shown.

Class I mutations impair protein production, being often nonsense mutations that generate premature stop codons leading to nonsense-mediated decay (NMD); Class II mutations affect CFTR processing causing the protein to misfold, leading to retention in the ER and early degradation through the ubiquitin-proteasome pathway; Class III mutations impair gating of the CFTR channel and are mainly mutations located in the NBDs; Class IV mutations cause decreased ion conductance, and localize mostly to the MSD's; Class V mutations reduce CFTR protein levels often by affecting splicing; Class VI mutations decrease the retention and stability of CFTR at the cell surface; and Class

VII mutations lead to a total absence of CFTR mRNA production and have been termed "unrescuable" mutations because they cannot be pharmacologically rescued (e.g. large gene deletions) [9], [27].

To add further complexity to this classification system, certain mutations may lead to more than one class of functional defect, as for example F508del results in class II and III defects [2], [28]. It has been proposed to group CFTR variants into theratypes according to their effect on the CFTR protein and in response to correctors and potentiators [12]. This way, unclassified variants can be provisionally assigned to theratypes on the basis of their effect on CFTR quantity and function in studies performed in cell lines.

Mutation specific therapies

Major efforts have identified strategies that rescue different defects of mutant CFTR. One of the major developments on this regard was the identification of its temperature sensitivity. Incubation at a lower temperature than the physiological 37^{o}C promotes the appearance of a fully-glycosylated mature form of CFTR [27] and was the first evidence that the protein can be rescued.

Mutation-specific therapies have become an important area of drug discovery for CF based on the classification of the mutations into classes [29]. For class I mutations, aminoglycoside antibiotics have been reported to supress premature termination codons by read-through leading to expression of full-length CFTR [27], [29]; For class II mutations, chemical, molecular or pharmacological chaperones, normally called correctors, were reported to stabilize protein structure and promote folding leading to the expression of the mutants at the PM. For class III and IV mutations, in which the CFTR is located at the PM, the modulation of these variants aims at activating the dysfunctional channel (either its gating or its conductance, respectively) with potentiators [9], [27], [29]. For class V mutations, splicing factors that promote normal exon inclusion or factors that promote abnormal exon skipping can increase levels of properly spliced transcripts. Single-stranded antisense RNA-based oligonucleotides can act as guide sequences to repair the targeted abnormal mRNA [9]. For class VI mutations compounds that enhance CFTR retention/ anchoring at cell surface will benefit these mutants [2]. Finally, for patients with unrescuable mutations, class VII mutations, the most straightforward approach is to target alternative non-CFTR anion channels to restore the ionic homeostasis of the epithelia, such as the calcium (Ca^{2+})-activated Cl^{-} channels (CaCCs) i.e., anoctamins 1 or 6 or some of the members of the SLC26 family of transporters [2], [9].

Three chemical compounds identified by high-throughput (HT) screening have led to new perspectives in CF treatment, particularly for class II correctors: lumacaftor (VX-809) and tezacaftor (VX-661) and for III mutations, potentiator ivacaftor (VX-770). Lumacaftor and tezacaftor are correctors that improve the conformational stability of CFTR bearing F508del, resulting in increased processing and trafficking to the cell surface. Ivacaftor is a potentiator that activates CFTR conductance when the protein is already at the PM [30]. Two of these drugs reached the market - Kalydeco (VX-770) and Orkambi (VX-770 + VX-809), being thus available for CF patients (**Figure 1.4**) [31].

Despite that 40-45% of patients (most of them F508del-homozygous) can already benefit from these approved therapies, there are still <50% of the patients for whom finding an effective treatment is still an unmet need.

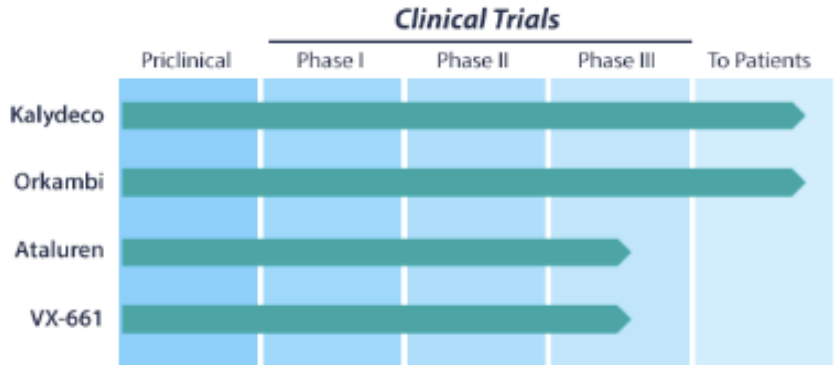


Figure 1.4. **Drugs already on clinical trials and drugs available to treat CF patients.**

1.2.4. Orphan mutations

Among the ~2,000 CFTR mutations, many of them are very rare variants, these are the so-called orphan mutations. For these mutations, the prediction of disease outcome is difficult, since the functional defect has not been defined. Many of these mutations may result in partial (residual) CFTR function and milder "atypical" forms of CF [25]. As each of these mutations affect very few patients worldwide, it is difficult not only to establish the diagnosis, but also assess the potential of the new mutation-based therapies. Nevertheless, some of these patients bearing these rare mutations are likely to respond to existing CFTR modulators. Thus, there is a need to do a molecular and functional characterization of these orphan mutations in order to establish validated compounds for mutation-based therapies [9], [25], [32].

1.3. Models to study CF

To study the many aspects of CF pathology, different model systems have been developed. To allow the study of CFTR in its natural environment – i.e. the membrane of a differentiated epithelial cell - many immortalized cell lines have been used [33]. Cell lines with an epithelial phenotype - with respect to polarization, tight junctions (TJs) and ion transport are desirable to study CF – have been engineered to express normal or mutant CFTR and can be used to the study of rare CFTR mutations or HT approaches to search for novel rescuing strategies (modulators or others). These cell lines have over-expression of CFTR and are normally quiescent, hence they do not present the complete phenotype of the parent tissue, decreasing its physiological relevance [34].

When more physiological models of airway epithelium are required, primary cultures of human nasal epithelial (HNE) or bronchial epithelial (HBE) cells are used. These cells can be obtained from nasal brushings, nasal polyps and lung explants or biopsies. When grown on porous supports at an air-liquid interface (ALI), they recapitulate many features of the native epithelium, hence, this model has been extensively used to study CFTR function and to test CFTR modulators. Nevertheless, good patient material is limited, it is time consuming and the reproducible differentiation can be difficult to achieve. Moreover, ALI cells are difficult to transfect with plasmids or infect with viral vectors [34], [35].

A variant procedure using primary intestinal organoids have been described. These are produced out of primary adult stem cells from the rectum crypts and can be cultured in matrigel for long times *in vitro* without genetic modifications. CFTR activation at the apical membrane by forskolin (Fsk) results in salt and fluid secretion into the organoid lumen and rapid organoid swelling. But this phenomenon

does not happen in organoids from patients with CF [9]. The magnitude of swelling can be correlated with CFTR activity, for this reason they can be used to study the effect of CFTR modulators, leading to the next step, testing the clinical benefit *in vivo*.

Although physiological relevant, the use of patient-derived materials needs to be complemented by cellular models – most mutations exist in compound heterozygosity and it is hard to ascertain if an observed effect comes from one or other allele. Thus, the production and use of novel cell lines is a requirement in the study of rare mutations.

2. Objectives of the present work

The main goal of the present work is to characterize CFTR orphan mutations at the cellular and functional level using novel cellular models and also, whenever available, to compare these results with those in patient-derived materials. We focused on very rare mutations many of which however occur in Portuguese CF patients, but which have not yet been characterized or likely misclassified. In addition, we also propose to assess the efficacy of existing CFTR corrective drugs on such CFTR mutations.

In order to achieve this goal, we propose the following specific objectives:

1) To generate stable cell lines expressing each mutant by:
    a. generating CFTR constructs carrying orphan mutations (P205S, L206W, R334W, R347P, I507del, R553P, R560S, L997F, H1079P, M1101K and D1152H) by site-directed mutagenesis using as a backbone CFTR cDNA cloned in the mammalian expression vector pcDNA5/FRT;
    b. sub-cloning each CFTR mutant cDNA into the lentiviral vector pLVX-Puro;
    c. using lentiviral transduction of CFBE cells to generate novel stable cell lines expressing each of the above CFTR mutants;
2) To assess processing, expression and localization of CFTR mutants, by Western blot (WB) and immunofluorescence (IF);
3) To assess function of CFTR mutants by assessing CFTR-mediated transepithelial Cl^- transport of polarized monolayers in Ussing chamber;
4) To test the efficacy of approved corrective drugs, by testing CFTR response in our newly created cellular models (as well as in patient-derived intestinal organoids).

Through the functional characterization of CFTR orphan mutations using our novel *in vitro* cellular systems and as well as organoids, primary cells and native tissues (whenever available and also studied in our lab), we will be able to confirm the diagnosis of many patients with a suspicion of CF and to get information on their prognosis. Moreover, by testing the response of these rare mutations to approved drugs in such systems, we can predict the *in vivo* response of patients carrying these orphan mutations to such drugs.

3. Materials and Methods

3.1. Generation of cell lines overexpressing wild-type and mutant CFTR

3.1.1. Plasmids and cDNAs

cDNA of human wild-type CFTR (wt-CFTR) was available in our lab cloned into pNUT and pcDNA™5/FRT vectors [36]. Each mutation under study was first introduced by site-directed mutagenesis into pcDNA™5/FRT carrying the full-length wt-CFTR cDNA (kindly given by Garry R. Cutting, M.D.) using KOD HOT start DNA polymerase (Novagen, Darmstadt, Germany). pNUT-CFTR-wt and pcDNA™5/FRT-CFTR-mutants (CFTR-Mut) are 8.1kb and 9.6kb vectors, respectively, both containing an ampicillin resistance gene, which was used for selection of transformed bacteria. Inserts from pNUT-CFTR-wt and pcDNA™5/FRT-CFTR-mutants where then subcloned into pLVX-Puro (Clontech, USA) vector. pLVX-Puro is a 8.1kb lentiviral vector with ampicilin and puromycin resistence genes, used to produce lentiviral particles to transduce cells and create stably transfected cell lines.

For cDNA and exon nomenclature, mutations are numbered based on the legacy name (Cystic Fibrosis Mutation Database [19]).

3.1.2. Mutagenesis

Each CFTR mutation in study was inserted into wt-CFTR by site-directed mutagenesis. The mutagenesis reactions were performed using the KOD Hot Start Kit (Novagen, USA) with the primers described in **Table 3.1**. In this PCR reaction, the primers are not completely complementary to the template sequence, having a mismatch at the selected mutation location. The PCR program can be found in **Table 3.2**. The PCR reaction results in a mixture of the template plasmid and the new plasmid containing the mutation. After confirmation of the amplification (by electrophoresis using a 1% agarose gel), the PCR products were incubated 1h at 37 ºC with *DpnI* (Invitrogen, USA), that hydrolyses methylated DNA. The template DNA, heavily methylated, is degraded, leaving the mutated DNA intact.

Competent bacteria were then transformed with the mutated DNA and grown in LB agar plates with 100 µg/mL ampicillin (selection antibiotic). The plasmid DNA of some colonies were extracted and purified and the mutation insertion was confirmed by DNA sequencing.

Table 3.1. **List of primers used for site-directed mutagenesis.**

Mutation		Sequence (5'- 3')
P205S	Forward	CGTGTGGATCGCTGCTTTGCAAGTGG
	Reverse	CCACTTGCAAAGCAGCGATCCACACG
L206W	Forward	GGATCGCTCCTTGGCAAGTGGCACTC
	Reverse	GAGTGCCACTTGCCAAGGAGCGATCC
R334W	Forward	CAAAGGAATCATCCTCTGGAAAATATTCACCACC
	Reverse	GGTGGTGAATATTTTCCAGAGGATGATTCCTTTG
R347P	Forward	GCATTGTTCTGCCCATGGCGGTCAC
	Reverse	GTGACCGCCATGGGCAGAACAATGC
I507del	Forward	CCATTAAAGAAAATATCTTTGGTGTTTCCTATG
	Reverse	CATAGGAAACACCAAAGATATTTTCTTTAATGG
R553P	Forward	CCATTAAAGAAAATATCTTTGGTGTTTCCTATG
	Reverse	CATAGGAAACACCAAAGATATTTTCTTTAATGG
R560S	Forward	GCAAGAATTTCTTTAGCAAGCGCAGTATACAAAGATGCTG
	Reverse	CAGCATCTTTGTATACTGCGCTTGCTAAAGAAATTCTTGC
L997F	Forward	CATATTTGACTTCATCCAGTTCTTATTAATTGTGATTGGAGC
	Reverse	GCTCCAATCACAATTAATAAGAACTGGATGAAGTCAAATATG
H1079P	Forward	CTTTGAAACTCTGTTCCCCAAAGCTCTGAATTTAC
	Reverse	GTAAATTCAGAGCTTTGGGGAACAGAGTTTCAAAG
M1101K	Forward	CAACACTGCGCTGGTTCCAAAAGAGAATAGAAATGATTTTTG
	Reverse	CAAAAATCATTTCTATTCTCTTTTGGAACCAGCGCAGTGTTG
D1152H	Forward	CTGTAAACTCCAGCATACATGTGGATAGCTTGATG
	Reverse	CATCAAGCTATCCACATGTATGCTGGAGTTTACAG

Table 3.2. **PCR program for the mutagenesis reaction.**

Program		
Temperature (^{o}C)	**Time**	**Number of cycles**
95	2 min	23
95	20 sec	
48	10 sec	
70	4 min	
4	pause	

3.1.3. Bacteria Transformation

100ng of DNA were added to a 200 µL aliquot of competent cells (the XL1-Blue bacterial strain (Stratagene, USA) was used to prepare competent cells). Bacteria were incubated 30min on ice followed by heat-shock 1.5 min at 42°C, incubated for 2 min on ice and then incubated in LB medium for 1h at 37°C at 220 rpm. After centrifugation, the supernatant was discarded and the pellet was ressuspended. This bacterial suspension was then plated into LB-agar plates (Sigma-Aldrich, USA) supplemented with 100 mg/ml ampicillin (selection antibiotic) and left growing overnight at 37°C at 220 rpm. The following day the plates were stored at 4^{o}C.

The selected colonies were used to inoculate LB medium supplemented with ampicillin and

cultures were grown overnight at 37ºC at 220rpm. The following day the plasmid DNA was extracted and purified.

3.1.4.Cloning

wt and mutant CFTR cDNAs were subcloned into pLVX-puro using the In-Fusion® HD Cloning Kit (Clontech, USA, 631187). pLVX with the insert was then used to produce lentiviral particles to transduce human cells and create stably transfected cell lines expressing either *wt* or mutant CFTR.

cDNAs from the original vectors were PCR amplified (**Table 3.3**) with primers designed to amplify CFTR and to create 15bp extensions at both the C- and N- terminal (using Primer Design tool for In-Fusion® HD Cloning Kit, Clontech, USA) (**Table 3.4**).

pLVX-Puro was linearized using the restriction enzyme *XhoI* (Thermo Scientific, USA) (3h at 37 ºC) to create sticky ends which are complementary to the primer extensions. Both linearized vector and *wt* or mutant inserts with extensions were spin-column purified using the NZYGelpure kit (NZYTech, Portugal). Then, the In-Fusion cloning reaction (In Fusion HD Cloning Kit, Clontech, USA) was performed and used to transform competent bacterial cells. The insertion was confirmed through colony PCR using CFTR specific primers (**Table 3.4**). The colony PCR reaction is a regular PCR reaction using DNA directly from bacterial colonies without extraction and purification. Cloning of the cDNA into the pLVX vector was also confirmed by restriction analysis with the enzyme *EcoRV* (Promega, USA) – detection of 2 bands with a different size from the empty vector when analysed by agarose gel electrophoresis was indicative of successful cloning. The full constructs were then sequenced (outsorced to StabVida).

Table 3.3. **PCR program for cDNA amplification wt and mutant CFTR.**

Program		
Temperature (ºC)	**Time**	**Number of cycles**
95	2 min	35
95	20 sec	
60	10 sec	
70	5 min	
4	pause	

Table 3.4. **Primers used for the amplification of each CFTR mutant and colony PCR reaction**.

Primers for cDNA ampification	
Name	**Sequence (5'- 3')**
Forward	GGACTCAGATCTCGAATGACATCACAGCAGGTCA
Reverse	GAAGCTTGAGCTCGACTAAGAGGCTGTGTCTGG
Primers for colony PCR	
Name	**Sequence (5'- 3')**
Ex2F (forward)	AAGGATACAGACAGCGCC
Ex5R (reverse)	GGAGACTAACAAGTTGTCC

3.1.5. Extraction and Purification of plasmid DNA

Plasmid DNA purification was performed using the NZYMiniprep kit (NZYTech, Portugal) based on the alkaline lysis of bacterial cells followed by adsorption of plasmid DNA into a silica gel-based spin column. Other impurities such as proteins, salts, oligos were washed away. In the end, the DNA is eluted in water.

Plasmid DNA concentration was assessed using a Nanodrop ND1000 Spectrophotometer (Thermo Scientific, USA) (absorbance at 260nm).

3.1.6. Plasmid cDNA Sequencing

Plasmid cDNA sequencing was performed by StabVida (Costa Caparica, Portugal) using the Sanger sequencing method. The primers used were specific for CFTR and are shown in **Table 3.5** and **Table 3.6**. Each CFTR mutant sequence was then compared with a CFTR-wt reference sequence (ENST00000003084.10) using the open-source software Geneious.

***Table 3.5*. List of primers used to confirm the insertion of mutations.**

Primers			
Mutation		**Name**	**Sequence (5'- 3')**
P205S	Forward	Ex4F	CTTCCTATGACCCGGATAA
L206W			
R334W		B2R	GGAAGGCAGCCTATGTGAGA
R347P			
I507del		B3R=R3R	AATGTAACAGCCTTCTGGGAG
R553P		Ex10F	GGCACCATTAAAGAAAATATCATCTT
R560S			
L997F		CF Ex15F	TGCAGTGATTATCACCAGC
H1079P		I1R	CTCACAGCAACTCAAACAAC
M1101K			
D1152H		Int17A	GCATATTTCCTCCAAACC

***Table 3.6.* List of primers used for sequencing the whole CFTR.**

Name		**Sequence (5'- 3')**
AC1L	Reverse	GAAACCAAGTCCACAGAAGGC
CMV	Forward	CGCAAATGGGCGGTAGGCGTG
Ex5F		CTCCTTTCCAACAACCTGAAC
B3R		AATGTAACAGCCTTCTGGGAG
C2R		AGCAGTATACAAAGATGCTG
D1R		GACAACAGCATCCACACGAA
E1R		AGATTCTCCAAAGATATAGC
Ex18F		AACTCCAGCATAGATGTGG
Ex22F		AGCAGTTGATGTGCTTGGC

3.2. Cell culture

3.2.1. Cell lines and culture conditions

The novel stable cell lines produced in this work (CFBE pLVX-P205S, CFBE pLVX-R334W, CFBE pLVX-R560S, CFBE pLVX-H1079P) were generated as described in **3.1.** by lentiviral transduction of the Cystic Fibrosis Brochial Epithelial (CFBE41o-) cell line. CFBE41o-, futher referred to as CFBE were developed from brochial epithelial cells from a F508del-CFTR homozygous CF patient [37].

Before producing stable cell lines and to test the novel constructs, transient transfections were performed using Human Embryonic Kidney (HEK 293T) cells [38], [39]. All cell lines were maintained at 37ºC in a humidified atmosphere of 5% (v/v) CO_2.

3.2.2. Transient transfections

Transient transfections using Lipofectamine 2000

Lipofection uses a cationic lipid to form an aggregate with the DNA, which is negatively charged, with these aggregates or liposomes being then internalized by the cells. HEK 293T cells were transfected using Lipofectamine 2000 (Invitrogen, USA).

The procedure consists in preparing two mixtures - one containing lipofectamine 2000 diluted in opti-MEM Reduced Serum Medium (Gibco, USA), and another containing the DNA also diluted in opti-MEM. The two dilutions were then mixed and incubated for 5min at room temperature. The DNA-lipofectamine complex was then added to the cells, which were seeded the day before (70-80% confluent). 24h post-transfection, the medium was changed to remove the complexes. 48h post-transfection, protein or RNA were extracted for further analysis.

Calcium Phosphate transient transfections

A precipitate containing calcium phosphate and DNA is formed slowly by mixing a HEPES-buffered saline solution with a solution containing calcium chloride and DNA. The precipitate adheres to the cell surface and the day after transfection should be visible black dots on the medium. This protocol was performed for the production of lentiviral particles used to transduce parental CFBE cells (see below).

3.2.3. Production of Lentiviral Particles

Lentiviral particles with pLVX-puro-CFTR-wt or pLVX-puro-CFTR-mutant cDNAs were produced in packaging cells - human embryonic kidney (HEK) 293T cells. $5x10^5$ cells were seeded per well in a 6-well plate containing Eagle's Minimum Essential Medium (EMEM, Lonza-BioWhittaker, Switzerland) plus 10% of Fetal Bovine Serum (FBS) (Gibco Life Technologies) and incubated for 24h at 37ºC, 5% CO_2. The next day, the cells in each well were transfected with 5 µg of pLVX-puro-CFTR, 4 µg of packaging plasmid pCMV-dR8.74psPAX2 and 0.4 µg of envelop plasmid VSV-G/pMD2.G. The transfection was performed using a calcium-phosphate transfection protocol. Cells were then incubated for 24h at 37ºC, 5% CO_2, after which the medium was changed to remove the transfection reagent. After an additional incubation for 48h at 37^{o}C, 5% CO_2, the media containing

the lentiviral particles was collected and the packaging cells were discarded. The lentiviral particles were immediately used or stored at -80ºC for further use.

3.2.4. Lentiviral infection – Production of stable cell lines

CFBE 41o- cells were seeded at a density of $4.0x10^5$ cells per well on a 6-well plate containing EMEM plus 10% FBS and were incubated for 24 h at 37ºC, 5% CO_2. Cells were infected with 1mL of medium containing pLVX-puro-lentiviral particles, 1mL of EMEM containing 10% FBS and the infection enhancer Polybrene (8 µg/mL) (Hexadimethrine bromide, Sigma-Aldrich, H9268-5G). Plates were centrifuged at 220rpm for 1h at 25ºC and then incubated for 24h at 37ºC, 5% CO_2.

The medium was changed to EMEM plus 10% FBS with 2 µg/mL of puromycin (Sigma-Aldrich, USA), which is half of the concentration needed to kill all the non-infected cells. The cells were incubated for 24h at 37ºC, 5% CO_2 and the medium was then changed to EMEM plus 10% FBS supplemented with 5 µg/mL of puromycin. The cells were kept in culture at 37ºC, 5% CO_2.

3.2.5. Treatment with CFTR modulators

VX-809

VX-809 or lumacaftor (Selleckchem, USA) is a CFTR corrector [40]. CFBE cells seeded in a 24-well plate were incubated with VX-809 3µM diluted in EMEM plus 0.1% v/v FBS. 24h post-treatment the protein was extracted for further assays.

VX-661

VX-661 or tezacaftor (Selleckchem, USA) is a CFTR corrector [41].CFBE cells seeded in a 24-well plate were incubated with VX-661 3µM diluted in EMEM plus 0.1% of FBS and after 24h the protein was extracted.

Cysteamine and Epigallocatechin gallate

Cysteamine (Sigma- Aldrich, USA), the reduced form of cysteamine, is an FDA-approved drug [42]. Epigallocatechin gallate (EGCG) (Sigma- Aldrich, USA) is a green tea flavonoid [43]. CFBE cells seeded in a 24-well plate (day before) were incubated with cysteamine 150µM alone or combined with EGCG 50µM.

3.2.6. Polarized cultures

To perform functional analysis in Ussing chambers, polarized cultures of CFBE cells were prepared. The cells were seeded on collagen IV (Sigma-Aldrich, USA) coated 12mm Snapwell filter (Corning, USA) inserts with 0.4 µm pore polyester membrane and 1.12 cm^2 surface area. Cells were seeded at a density of $3x10^5$ cells per filter on the apical side (top side) and the filters were maintained in liquid-liquid interface. The medium was changed every other day and the transepithelial electrical resistance (TEER) was measured using a volt-ohmmeter (Milicell-ERS, Millipore, MERS00001). Cells took 2-7 days to polarize. When the TEER value was above 600 Ω/cm^2, the experiments were performed.

3.3. Protein Analysis

3.3.1. Immunofluorescence

CFBE cells grown on glass coverslips in 24-well plates were washed 3 times with PBS supplemented with calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) (PBS^{++}), and fixed for 10 min with paraformaldehyde (PFA) 4% (v/v) (Merck Millipore, USA). Then, the cells were washed 3 times with PBS^{++}, permeabilized for 10 min with Triton X-100 0.1% (v/v) (Amersham Biosciences, UK) and washed again with PBS^{++}. After this, cells were incubated at room temperature with primary antibodies (see table 7 below) diluted in PBS with Bovine serum albumin (BSA) (Sigma-Aldrich, USA) 1% (w/v) for 1h. The cells were then washed 3 times with PBS^{++} and incubated at room temperature in the dark with secondary antibody (**Table 3.7**) diluted in PBS with BSA 1% (w/v) for 1h. The cells were again washed 3 times with PBS^{++} and incubated 10 min in the dark with Hoechst 33342 Fluorescent Stain (Life Technologies, USA). The cells were washed one last time with PBS^{++} and the cover slips were mounted in glass slides with mounting solution (0,5% n-propyl-gallate; 1 mL PBS 10X; 9 mL glycerol for microscopy) and sealed. Immunofluorescence staining was observed and acquired in a Leica DMI 6000B fluorescence microscope.

Table 3.7. **Primary and secondary antibodies used in immunofluorescence assays.**

Target	Dilution	Host	Company	Reference
Primary antibodies				
CFTR	1:200	Mouse	CFF	570
β-catenin	1:200	Rabbit	Abcam	Ab32572
Secondary antibodies				
Alexa fluor 488 (Anti-mouse)	1:500	Donkey	Life Technologies	A21202
Alexa fluor 568 (Anti-rabbit)	1:500			A10042

3.3.2. Image acquisition, processing and analysis

Images were acquired with a Leica DMI6000B system equipped with a metal halide light source (EL6000) and a DFC365 FX CCD camera (Leica). Fluorescence was assessed in three different channels: Alexa Fluor 488 (excitation 490-510, emission 520-550) and Alexa Fluor 568 (excitation 515-560, emission >590) and Hoechst (excitation 340– 380, emission 450–490).

3.3.3. Western Blot

Cell lysis and protein extraction

Lysates from cells grown in 24-well plates were prepared by washing cells twice with cold PBS and adding the appropriate volume of sample buffer (31.25 mM Tris HCl (Sigma, USA) pH 6.8; sodium dodecyl sulfate (SDS) 1.5% (v/v) (Gibco, USA); glycerol 5% (v/v) (Sigma, USA); bromophenol blue 0.02% (w/v) (Sigma-Aldrich, USA); dithiothreitol (DTT) 50 mM (Sigma, USA)). Benzonase (Sigma-Aldrich, E1014) 25 U/mL was added to shear the DNA, in the presence of $MgCl_2$ 3mM. Cells were scraped to collect the lysates.

Western Blot

Samples were separated by SDS-PAGE using a 7% (w/v) separating gel (TrisHCl 375 mM pH 8.8; acrylamide 7% (v/v) (Bio-Rad, USA); glycerol 0.1% (v/v); SDS 0.1% (v/v); ammonium persulfate (APS) 0.075% (v/v) (Bio-Rad, USA); tetramethylethylenediamine (TEMED) 0.06% (v/v) (Sigma- Aldrich, USA)) and a 4% stacking gel (Tris HCl 125 mM pH 6.8; acrylamide:bisacrylamide 4% (v/v); glycerol 0.1% (v/v); SDS 0.1% (v/v); APS 0.075% (v/v); TEMED 0.08% (v/v)) gels. Samples run on Tris-Glycine-SDS buffer (Bio-Rad, USA) for 90 to 210min at 65 to 120V. The next step was the transfer onto polivinylidene difluoride (PVDF) membranes (Merck Millipore, USA) using Tris-Glycine buffer (Bio-Rad, USA), at 400mV for 1h30min. The membranes were blocked for 1h with 5% (w/v) skimmed milk (Nestlé, Molico, Switzerland) in PBS supplemented with Tween 20 0.1% (v/v) (PBS-T) and then incubated overnight at 4ºC with primary antibodies (**Table 3.8**) diluted in 5% milk in PBS-T. After washing 3 times with PBS-T the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody 1h at room temperature.
Chemiluminescent detection was performed using Chemidoc XRS plus analyser (BioRad, USA) using a 1:1 mixture of peroxide:luminol/enhancer solution (BioRad, USA). Quantification of the intensity of the bands was performed using the ImageLab software (Bio-Rad, USA).

Table 3.8. **Primary and Secondary antibodies used in western-blot assays.**

Target	Dilution	Host	Company	Reference
Primary antibodies				
CFTR	1:3000	Mouse	CFF	596
Calnexin	1:3000	Mouse	BD Transduction Lab	610523
Secondary antibodies				
(H+L)-HRP Conjugate (Anti-mouse)	1:3000	Goat	Bio-Rad	170-6515

3.4. Functional Analysis

3.4.1. Ussing chamber

CFBE cells overexpressing each CFTR variant were seeded onto previously collagen IV-coated snapwell filters at a density of $3x10^5$ cells per filter. Transepithelial electrical resistance (TEER) was routinely measured using a volt-ohmmeter (see above).

For assessment of transepithelial transport, the basolateral surface of CFBE cells was continuously perfused with Ringer solution (NaCl 145 mM, KH_2PO_4 0.4 mM, K_2HPO_4 1.6 mM, D-glucose 5 mM, $MgCl_2$ 1 mM, Ca-gluconate 1.3 mM) and the apical surface with a low Cl^- Ringer solution (NaCl 38 mM, KH_2PO_4 0.4 mM, K_2HPO_4 1.6 mM, D-glucose 5 mM, $MgCl_2$ 1 mM, Ca-gluconate 1.3 mM).

Following a 20 min equilibration period, baseline values were recorded. Values were then recorded following the sequential addition of forskolin (2 μM) and IBMX (100 μM) - CFTR agonists - at the apical side, the CFTR potentiators VX-770 or genistein (50 μM), and finally the CFTR inhibitor GlyH101 (30 μM).

Values for the transepithelial voltage (V_{te}) were referred to the serosal surface of the epithelium. Transepithelial resistance (R_{te}) was determined by applying intermittent (1s) current pulses (0.5 μA). The equivalent short-circuit current (I_{sc}) was calculated according to Ohm's law ($I_{sc}=V_{te}/R_{te}$), after appropriate correction for fluid resistance.

3.4.2. Forskolin-induced swelling (FIS) assay

The FIS assay was carried out as previously described [44]. Isolation of crypts and organoids preparation was performed by Nikhil T.A. according to [44], [45]. Rectal CF organoids (Passage 3-25), from a 7-9-day-old culture were seeded in a pre-warm 96-well plate (Thermo Fisher Scientific, USA) with 5μl 50% matrigel containing approximately 20-60 organoids immersed in 50μl complete medium. The organoids were treated with the corrector VX-809 (3 μM) which was prepared in complete media. Potentiators or stimulators were prepared in DMEM-F12. One day after, the organoids were incubated with calcein green (3μM) (Invitrogen, USA) for 30 min. After calcein green staining, forskolin was added with and without potentiators (VX-770) at different concentrations. Live cell imaging was performed on a Leica DMI 6000B Fluorescence microscope (5x objective) for 120 min at 37 ^{o}C.

Quantification of forskolin-induced swelling

Forskolin-induced swelling was quantified using Cell profiler software. The area under the curve (AUC; t=60; baseline=100%) was calculated using GraphPad Prism version 5.01. A paired t-test was used to calculate statistical differences.

3.5. Statistical analysis

Data are presented as a mean and standard error of the mean (SEM). When necessary, Statistical Analysis Student's t-test for unpaired samples was performed, with $p<0.05$ considered as the level of statistical significance.

4. Results

4.1. Characterization of CFTR orphan mutations

In the present study we studied the following CFTR mutations (**Table 4.1**):

Table 4.1. **List of CFTR orphan mutations in study (ordered by amino acid number).**

MUTATION	cDNA NAME	PROTEIN NAME	LOCATION	DOMAIN
P205S	c.613C>T	Pro205Ser	Exon 6a	MSD1 (TM3)
L206W	c.617T>G	Leu206Trp	Exon 6a	MSD1 (TM3)
R334W	c.1000C>T	Arg334Trp	Exon 7	MSD1 (TM6)
R347P	c.1040G>C	Arg347Pro	Exon 7	MSD1 (TM6)
I507del	c.1519_1521delATC	Ile507del	Exon 10	NBD1
R553P	c.1790G>C	Arg553Pro	Exon 10	NBD1
R560S	c.1680A>C	Arg560Ser	Exon 12	NBD1
L997F	c.2991G>C	Leu997Phe	Exon 17a	MSD2 (TM9)
H1079P	c.3236A>C	His1079Pro	Exon 17b	MSD2 (ICL4)
M1101K	c.3302T>A	Met1101Lys	Exon 17 b	MSD2 (TM11)
D1152H	c.3454G>C	Asp1152His	Exon 18	Linker MSD2-NBD2

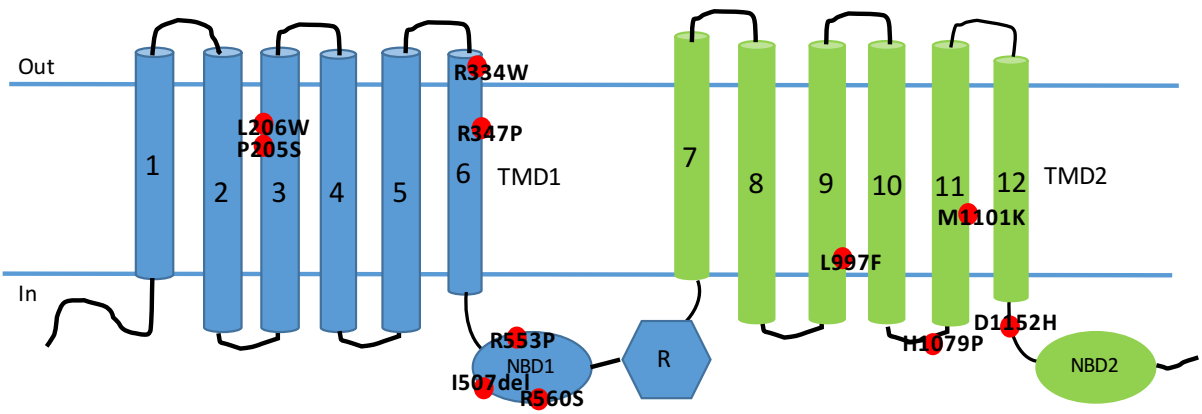


Figure 4.1. **Schematic representation of the location of each CFTR mutation in study on the CFTR protein.**

In order to characterize these orphan mutations (**Table 4.1**) at cellular and functional levels and to test their responsiveness to already approved corrective drugs, it was necessary to develop novel cellular models overexpressing each mutant CFTR.

CFTR constructs bearing each CFTR mutation were generated by site-directed mutagenesis using the wt-CFTR cDNA cloned into pcDNA5 mammalian expression vector as a backbone and sub-cloned into the lentiviral vector pLVX-Puro (**Figure 4.2**). In the end, the sequence of all constructs was confirmed by sequencing (see **Methods**). In order to test the newly created constructs and before the production of stable cell lines, HEK 293T cells were transiently transfected with each of the constructs. HEK293T cells were also used to produce lentiviral particles for each construct. These lentiviral particles were then used to transduce CFBE cells (a human bronchial epithelial cell line, thus a physiologically relevant cell model) and thus obtain stable cell lines expressing each of the CFTR mutants. CFBE cells were originally isolated from the bronchial epithelium of CF patients and immortalized, they do not express endogenous CFTR [46] and thus after transduction will express only the specific CFTR variant.

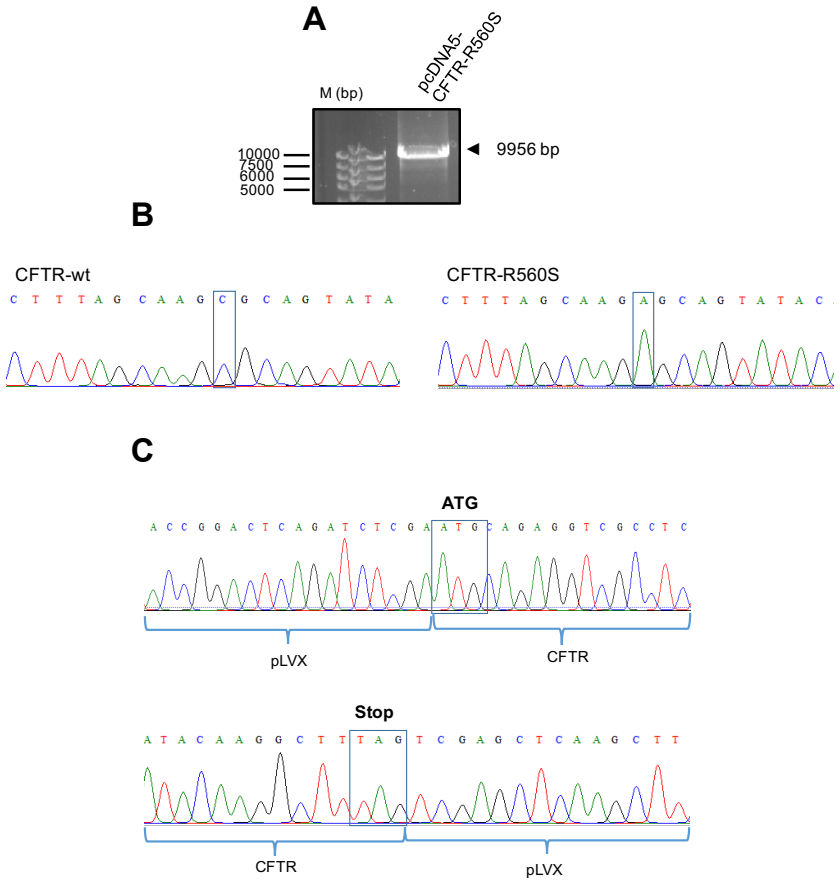


Figure 4.2. **Cloning of R560S-CFTR into pLVX-puro after mutagenesis. (A)** By site-directed mutagenesis the mutation R560S-CFTR was inserted into the pcDNA5-CFTR-wt vector. **(B)** Results of the sequencing used to confirm the insertion of the R560S mutation. **(C)** Results of the sequencing used to confirm the cloning of the R560S-CFTR sequence into the pLVX-Puro vector. The same approach was applied to develop the other cell lines described in this study.

Expression and processing CFTR mutants was assessed by WB. Two of the mutations (P205S and R560S) analysed were further analysed in terms of function by Ussing chamber and immunofluorescence to assess protein intracellular localization (only R560S).

The results obtained for each mutation are described below, using the same order with which they are listed in the table above.

4.1.1.P205S mutation

i) Assessment of P205S-CFTR protein processing

P205S is a missense substitution with a change from a proline residue to a serine residue at position 205, resulting from a C to T transition at position 613 in exon 6a (**Table 4.1**). It was first found in 4 Spanish CF patients and it is associated with a mild CF phenotype characterized by retention of some pancreatic function (pancreatic sufficiency). Its allele frequency in North-America and Europe is 0.02% [47] being more frequent in France and the US [19], [48]. Studies in Hela and HEK 293T cell lines showed that the mutation likely causes a defect in processing but has some residual CFTR function [49], [50].

To assess whether P205S affects CFTR processing, total protein was extracted from both HEK293T cells transiently transfected with pcDNA5-CFTR-P205S and from a novel CFBE cell line produced here, stably expressing P205S-CFTR. For this purpose, we performed WB assays to assess the presence of CFTR immature (band B) and mature (band C) forms. Similar results were obtained for both transient and stable cells (**Figure 4.3 A, B**). The protein resulting from wt-CFTR results in the mature (fully-glycosylated) form and in the immature (core-glycosysated) form. The P205S mutation results in the appearance of the immature form, similar to the F508del mutation, but also in a faint band C (**Figure 4.3 A, B**), indicating that they only a portion of CFTR reachs the cell surface. This result suggests that this CFTR mutation leads to a defect in CFTR processing (**Figure 4.3 C, E**). Though in HEK 293T cells it is possible to see a very faint band C that is not visible in CFBE cells probably due to the lower expression (**Figure 4.3 A**).

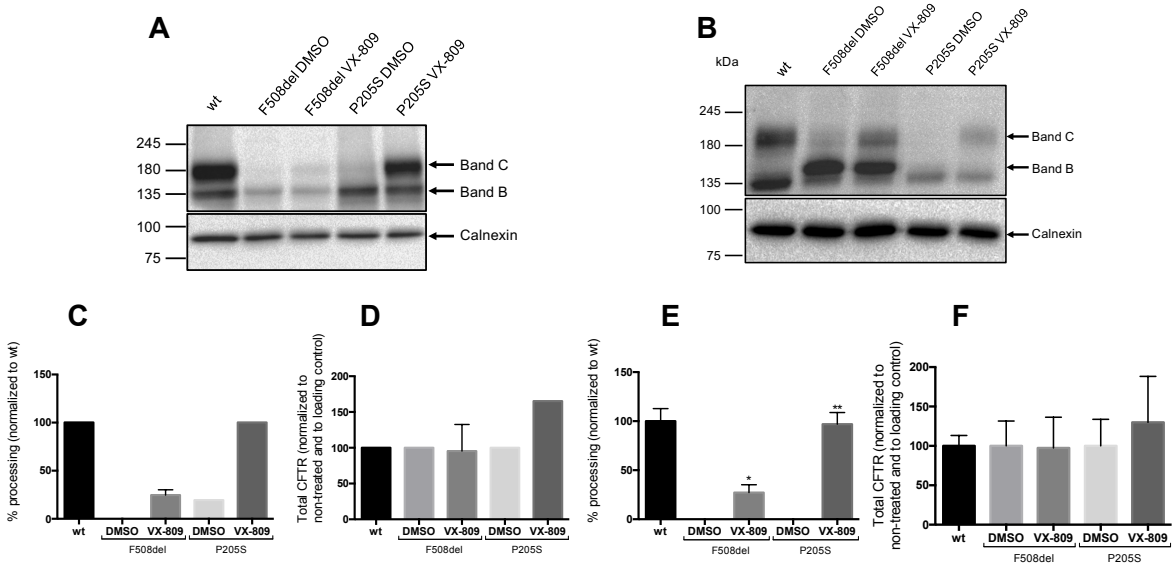


Figure 4.3. **Assessment of the P205S CFTR mutation protein processing and the effect of VX-809 using WB assays.** **(A)** Representative WB analysis of HEK293T cells transiently transfected with pcDNA5-CFTR-wt, pcDNA5-CFTR-F508del or pcDNA5-CFTR-P205S, and treatment with VX-809 (3 μM) and DMSO (0.1% v/v). **(B)** Representative WB analysis of CFBE cells stably expressing P205S-, F508del- or wt-CFTR, and treatment with VX-809 (3 μM) and DMSO (0.1% v/v). **(C)** Graph showing quantification of stably transfected CFBE cells. **(D)** Graph showing quantification of stably transfected CFBE cells (n=3). **(E)** Graph showing quantification of HEK293T transiently transfected with pcDNA5-CFTR-wt, pcDNA5-CFTR-F508del or pcDNA5-CFTR-P205S. For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed. Data were normalized to the efficiency of processing of wt-CFTR and is shown as mean ± SEM (n=3). and **(F)** Graph showing quantification of HEK293T transiently transfected with pcDNA5-CFTR-wt, pcDNA5-CFTR-F508del or pcDNA5-CFTR-P205S (n=1). Images were acquired using ChemiDoc XRS+ imaging system BIO-RAD and processed using Image lab 4.0 software. * P<0.05 was considered as significant.

We also tested the efficacy of the VX-809/Lumacaftor, a corrector drug approved (in combination with VX-770), in rescuing P205S-CFTR. For this purpose, we produced and analysed CFBE cells stably expressing P205S-CFTR and compared them with those expressing F508del- (used as a positive control for the compound VX-809 since it shows correction resulting in the appearance of band C), and wt-CFTR. Cells were incubated for 24h with VX-809 3 μM or DMSO 0.1% v/v (as a vehicle control) and WB was performed to assess whether the corrector was able to promote the appearance of mature P205S-CFTR (**Figure 4.3**). When treated with the corrector VX-809, mature CFTR was detected for both F508del- and P205S-CFTR, but more intensely in the latter (**Figure 4.3 A, B**). Again, similar results were obtained for both the cell lines. This result indicates that the P205S-CFTR mutation as a processing defect that can be rescued by the corrector VX-809.

ii) Assessment of P205S-CFTR function and effect of modulators

We also assessed the functional consequences of the P205S mutation (in collaboration with Iris Silva) by studying the transepithelial transport in polarized monolayers of CFBE cells stably expressing this mutant by using Ussing Chamber. Cells were stimulated with forskolin 2 μM and 3-isobutyl-1-methylxantine (IBMX) 100 μM and with a CFTR potentiator (either VX-770 3 μM or Genistein 25 μM) in order to detect CFTR function. Results showed that activation of cAMP-dependent CFTR-mediated Cl^- secretion with forskolin 2 μM and IBMX 100 μM elicited a typical lumen-negative response in CFBE cells expressing wt-CFTR, that was further potentiated by treatment with genistein and inhibited with the CFTR inhibitor GlyH101 30 μM (**Figure 4.4 A**). A similar approach was performed with the cells expressing the P205S mutation, showing some Cl^- secretion under potentiation with either VX-770 3 μM or genistein 25 μM alone (**Figure 4.4 B, D**), this lead to the conclusion that this mutation has some residual function. Treatment with VX-809 3 μM (in combination with potentiators) increased CFTR-mediated Cl^- secretion, however quite reduced (2.14 $\mu A/cm^2$) when compared to with wt-CFTR (7.5 $\mu A/cm^2$) (**Figure 4.4 C, E**). The combination VX-809+VX-770 did not produce a further increase in Cl^- secretion.

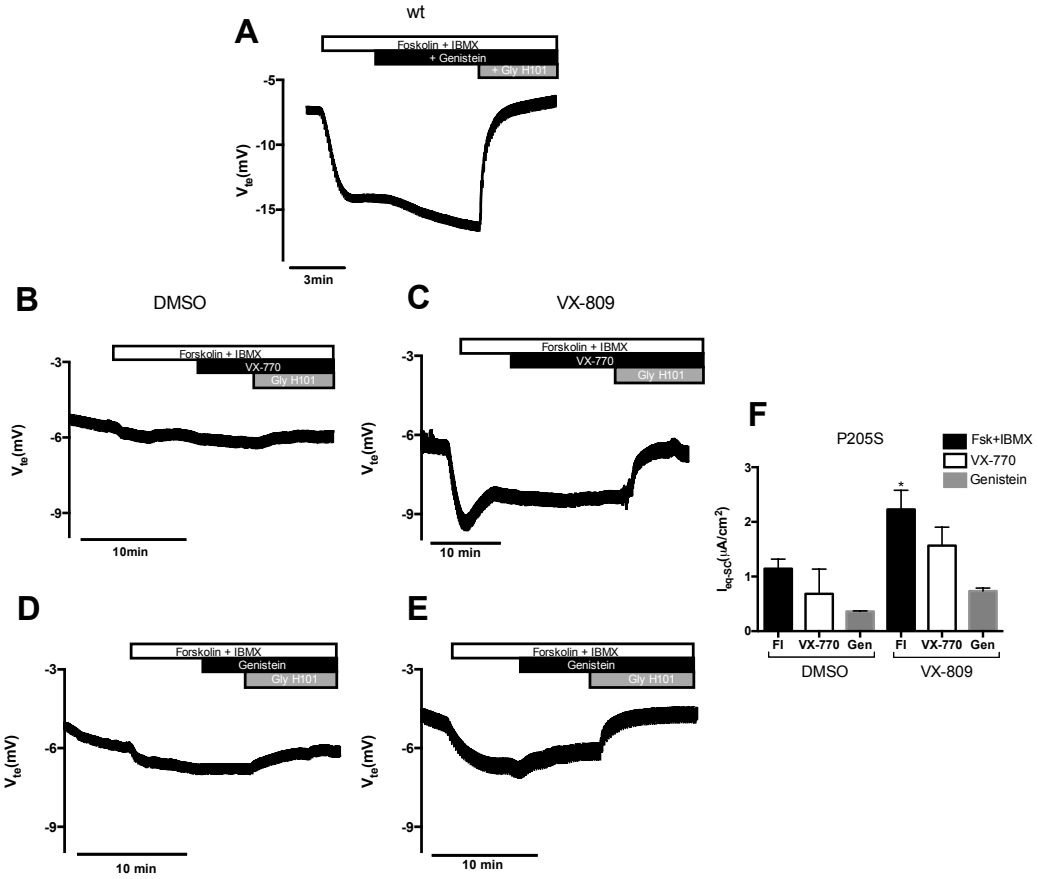


Figure 4.4. **Original Ussing Chamber (open-circuit) recordings showing transepithelial voltage measurements (V_{te}) obtained from CFBE stably expressing wt- or P205S-CFTR. (A)** CFBE stably transduced with wt-CFTR. **(B)** CFBE cells stably transduced with P205S-CFTR, pre-incubated for 24h with DMSO (0.1% v/v) (vehicle control) and under potentiation with VX-770. **(C)** CFBE cells stably transduced with P205S-CFTR, pre-incubated for 24h with 3 μM of VX-809 and under potentiation with VX-770. **(D)** CFBE cells stably transduced with P205S-CFTR, pre-incubated for 24h with DMSO (0.1% v/v) (vehicle control) and under potentiation with Genistein. **(E)** CFBE cells stably transduced with P205S-CFTR, pre-incubated for 24h with 3 μM of VX-809 and under potentiation with Genistein. **(F)** Graph showing the quantification of the current (I_{eq-sc}) after apical application of forskolin+IBMX and potentiators, either genistein or VX-770. A low chloride ringer solution was used at apical side to stablish a Cl^- gradient. Negative transepithelial voltage (V_{te}) deflections were observed following the application of apical forskolin (2 μM) and IBMX (100 μM) or with either VX-770 (3 μM) (B and C) or genistein (25 μM) (D and E). The latter are fully reverted by application of the specific CFTR inhibitor GlyH101 (30 μM). * $P<0.05$ was considered as significant.

From these results we were able to: (i) show that studies with the HEK 293T and the CFBE cell lines lead to similar observations, indicating that HEK 293T cells are also valid as as a model to study CF; (ii) determine that the P205S orphan mutation has a processing defect, (iii) showing, however some mature protein (iv) the trafficking defect caused by P205S can be corrected by VX-809 (96% of wt-CFTR processing) and (v) P205S-CFTR rescued with VX-809 has some Cl^- secretion that is not potentiated by VX-770. Results from our lab in rectal biopsies and organoids for this mutation showed some residual function (unpublished data by Iris S. and Nikhil T.A.). This supports our functional results from Ussing chamber and allowed us to classify this mutation as a class IV mutation.

4.1.2.L206W mutation – assessment of protein processing

L206W mutation is a missense mutation caused by a T>G transversion at cDNA position 617 located in exon 6a, resulting in the replacement of a leucine residue by a tryptophan one at position

206. This CF-causing mutation was identified in two unrelated CF patients from Southern France (allele frequency 0.19%)[19], [47]. This mutation is associated with pancreatic sufficiency (PS) and residual Cl^- secretion and has been classified as a class II mutation in studies performed in HeLa cells [51]–[54].

To study how this mutation affects the processing of CFTR, HEK293T cells were transiently transfected with cDNA encoding L206W-, F508del- and wt-CFTR proteins. Total protein was extracted and the presence of immature and mature forms of CFTR was assessed by WB. Results showed that CFTR bearing L206W appears both in its mature (band C) and immature (band B) forms (**Figure 4.5 A**). However, the efficiency of processing was 18% (normalized to wt-CFTR) thus evidencing a defect in processing/trafficking. (**Figure 4.5 B**).

We also investigate if the corrector VX-809 was able to promote the increase of band C for L206W-CFTR. For this HEK 293T cells transiently transfected with pcDNA5-CFTR-wt, pcDNA5-CFTR-F508del (positive control for the compound VX-809 since it shows correction resulting in the appearance of band C) or pcDNA5-CFTR-L206W. 24h after transfection, cells were incubated for 24h with VX-809 3 μM or DMSO 0.1% v/v (vehicle control). CFTR processing was assessed by WB as above (**Figure 4.5 A**).

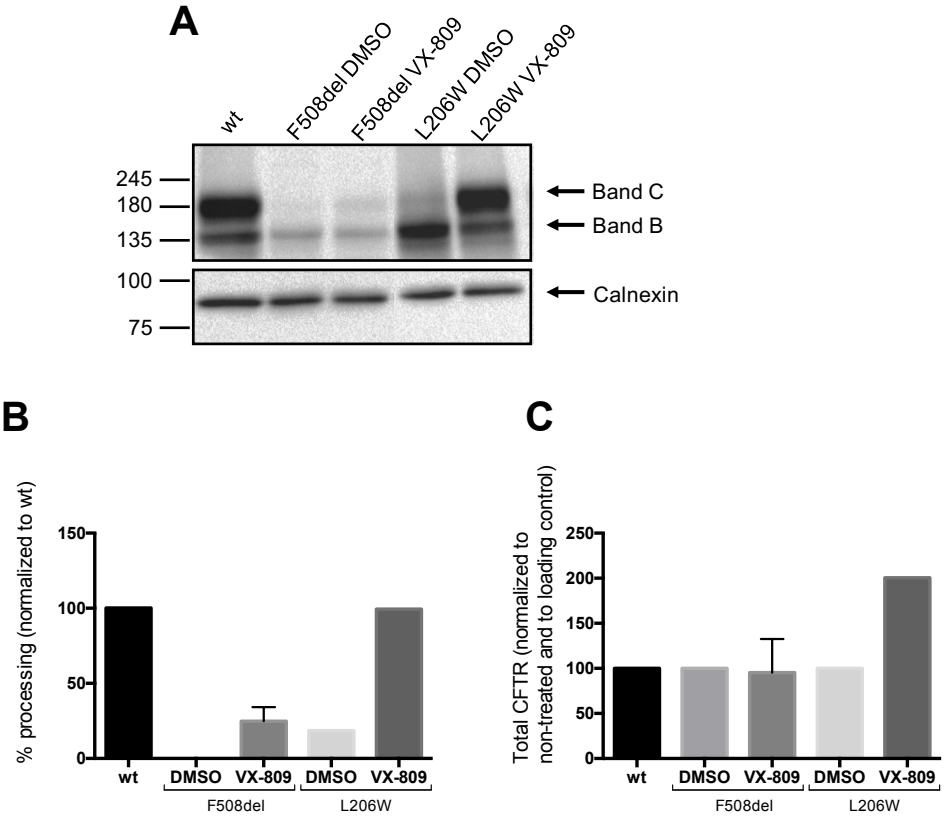


Figure 4.5. **Effect of VX-809 on processing of L206W-CFTR and F508del- (control) CFTR protein assessed by WB. (A)** Representative WB analysis of HEK293T cells transient transfected with pcDNA5-CFTR-wt, pcDNA5-CFTR-F508del or pcDNA5-CFTR-L206W, following treatment with VX-809 (3 μM) and DMSO (0.1% v/v). **(B)** For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vc total CFTR expressed. Data were normalized to the efficiency of processing of wt-CFTR (n=1). **(C)** For each condition, densitometry was used to calculate the total amount of CFTR protein, normalized to the loading control and to the amount of CFTR in the non-treated sample. Images were acquired using ChemiDoc XRS+ imaging system BIO-RAD and processed using Image lab 4.0 software.

After treatment with CFTR modulator VX-809, there was an increase in the quantity of L206W-CFTR protein in the mature form (band C more intense) (efficiency of processing increased from 18% to 99% normalized to wt-CFTR). From these results we were able to determine that L206W has a processing defect that was corrected by the VX-809 modulator. However since this mutation showed some mature protein, functional studies are needed to be able to classify this mutation.

The results obtained for both P205S and L206W strongly indicate that the TM3 is a critical region for CFTR processing. As the amino acid residue proline is rigid, it is probably stabilizing the structure, this way, when is substituted by a residue of serine it leads to defects in processing. The opposite occurs to the L206W, when a leucine residue is replaced by a tryptophan residue, an aromatic amino acid, it leads to destabilization of the structure, again leading to defects in processing.

4.1.3. R334W mutation – assessment of protein processing

R334W is a missense mutation firstly identified in 1991 [55], that results from a C>T transition at cDNA position 1000 located in exon 7, leading to the exchange of an arginine residue to a tryptophan residue at position 334, with an allele frequency of 0.3% [19], [47]. Expression studies in epithelial cells carrying this mutation showed that R334W-CFTR is correctly processed, but has a reduced ability to transport Cl- and the inclusion in class IV mutation has been suggested [56], [57]. Clinical data demonstrates that R334W in homozygosity is associated with a pancreatic sufficient (PS) phenotype, but, when in heterozygosity, can also be a pancreatic insufficient mutation, depending on the mutation present in the other allele [58]. Its allele frequency somehow excludes its inclusion as an orphan mutation, but these contradictory findings suggested its inclusion in this work.

To assess the effect of R334W on CFTR processing, total protein was extracted from a novel CFBE cell line developed in this work, which stably expresses R334W-CFTR. A Western Blot was performed to assess CFTR expression and processing (**Figure 4.6 A**).

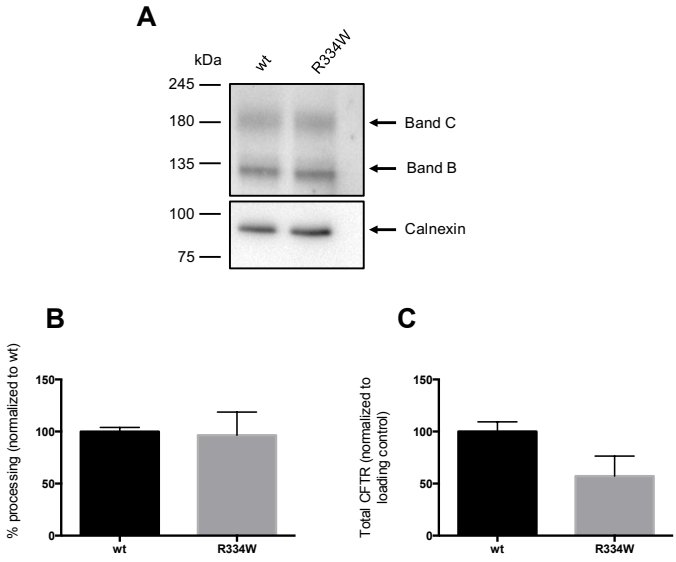


Figure 4.6. **Western Blot analysis of R334W-CFTR protein expression. (A)** Representative WB analysis of CFBE cells stably expressing R334W-CFTR. **(B)** For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed. Data were normalized to the efficiency of wt-CFTR processing and is shown as mean ± SEM (n=2). **(C)** For each condition densitometry was used to calculate the total amount of CFTR protein, normalized to the loading control and is shown as mean ± SEM. Images were acquired using ChemiDoc XRS+ imaging system BIO-RAD and processed using Image lab 4.0 software.

As shown in **Figure 4.6 A**, R334W-CFTR was detected in its immature (band B) and mature (band C) form, similarly to wt-CFTR. This result confirms that R334W-CFTR is correctly processed in human bronchial epithelial cells (**Figure 4.6 B**). The results obtained here suggest that R334W mutation can be either a class III or a class IV mutation. According to studies from our lab in patient-derived materials (Nikhil T.A., unpublished data), this mutation has residual function, being classified as a class IV mutation. However, functional studies are needed to confirm this classification.

4.1.4. R347P mutation – assessment of protein processing

The R347P mutation was first reported on a chromosome of North-American origin, having a frequency of 0.37% in North-America and Europe. The mutation results in a substitution of an arginine residue by a proline residue at position 347 caused by a transversion of a G to C at cDNA position 1040 located in exon 10 [59], [60]. Clinical data suggest that R347P mutation causes PI in most of the cases, being classified as a severe mutation [61]. Studies using Fisher rat thyroid (FRT) cells showed that expression of R347P generates the mature, fully-glycosylated form of the protein with significant apical membrane Cl^- channel function. However, the magnitude of the current generated was reduced [62]. Furthermore, studies in rectal organoids showed that this mutation has residual Fsk-induced swelling (FIS) but it is not responsive to VX-770 treatment [44].

To study whether this mutation disrupts the processing of CFTR, total protein was extracted from HEK 293T cells transiently transfected with cDNA encoding wt-, F508del- and R347P-CFTR. WB was performed as above (**Figure 4.7**).

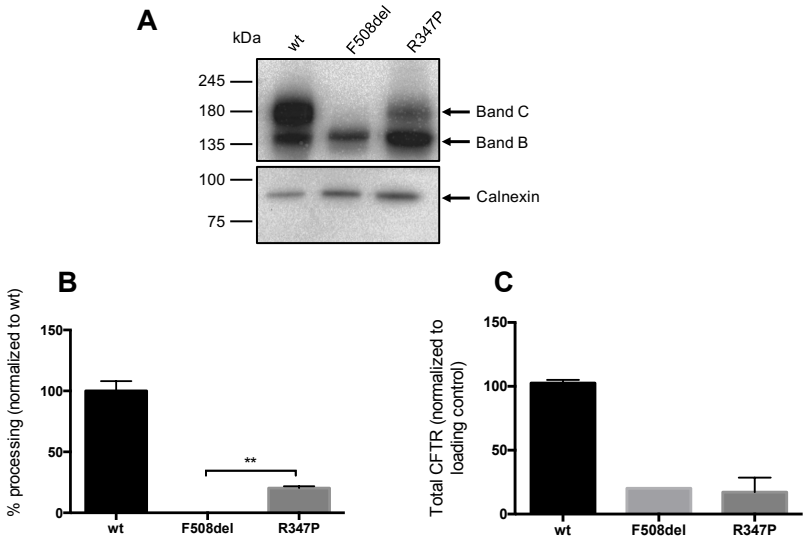


Figure 4.7. **Western Blot analysis of R347P-CFTR protein expression. (A)** Representative WB analysis of HEK 293T cells transient transfected with pcDNA5-CFTR-wt, pcDNA5-CFTR-F508del or pcDNA5-CFTR-R347P. **(B)** For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed. Data were normalized to the efficiency of wt-CFTR processing and is shown as mean ± SEM (n=3). **(C)** For each condition densitometry was used to calculate the total amount of CFTR protein, normalized to the loading control and is shown as mean ± SEM (n=3). Images were acquired using ChemiDoc XRS+ imaging system BIO-RAD and processed using Image lab 4.0 software. * $P<0.05$ was considered as significant.

As shown in **Figure 4.7A**, R347P-CFTR is present in both in the mature (band C), fully-glycosylated and in the immature (band B) forms, similarly to wt-CFTR and in contrast to F5808del-CFTR which only generates the immature form. From these results we were able to: (i) understand that R347P-CFTR does not cause a defect in processing (**Figure 4.7 B**); and (ii) functional data from

our lab on patient-derived materials (Nikhil T.A., unpublished data) suggest that this mutation has a functional defect which however does not totally abolish function. Altogether, these data suggest that R347P is a class IV mutation. However, further functional studies are necessary to confirm the classification.

4.1.5.I507del mutation – assessment of protein processing

I507del is a deletion of the codon ATC between cDNA positions 1519-1521 in exon 10, causing the deletion of the isoleucine residue at position 507 (allele frequency of 0.45%) [47]. Studies in COS-7 (green monkey kidney) and HEK293T cells have showed that this mutation causes a severe defect in CFTR processing and trafficking, characteristic of a class II mutation. Also, C3, C4 and C18 correctors were not able to restore the processing of CFTR bearing this mutation [30]. Clinical data suggest that most of the patients with this mutation are pancreatic insufficient [47].

In order to understand how this mutation affects CFTR, cDNA encoding wt-, F508del- and I507del-CFTR proteins were transiently transfected into HEK293T cells. Presence of mature (band C) and immature (band B) forms of CFTR was assessed by WB. The I507del mutation results only in the appearance of the immature form, similarly to F508del (**Figure 4.8A, B**), indicating a major defect in CFTR processing.

We next investigated if corrector VX-809 was able to promote the rescue of I507del-CFTR. To this end, HEK 293T cells transiently transfected with pcDNA5-CFTR-wt, pcDNA5-CFTR-F508del (used as a control) or pcDNA5-CFTR-I507del, 24h after transfection, were incubated for 24h with VX-809 3 μM or DMSO 0.1% v/v (as a vehicle control). Total protein was extracted and it was performed a WB (**Figure 4.8 A**).

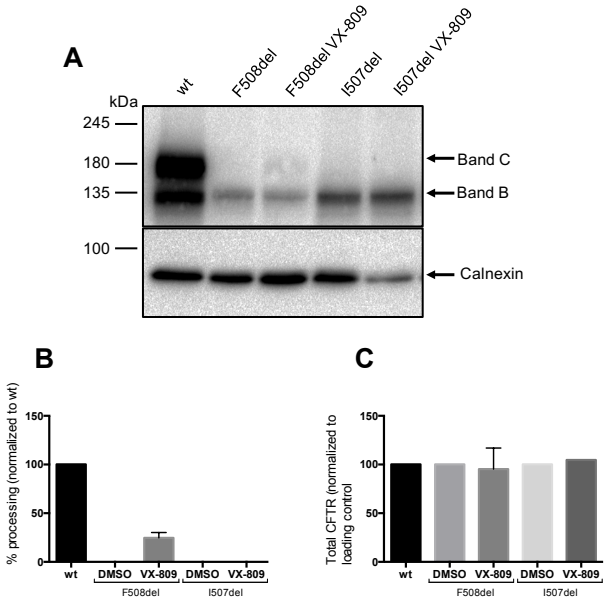


Figure 4.8. **Effect of VX-809 on processing of I507del-CFTR and F508del- (control) CFTR protein assessed by WB. (A)** Representative WB analysis of HEK 293T cells transient transfected with pcDNA5-CFTR-wt, pcDNA5-CFTR-F508del or pcDNA5-CFTR-I507del, following treatment with VX-809 (3 μM) and DMSO (0.1% v/v). **(B)** For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed. Data were normalized to the efficiency of processing of wt-CFTR (n=1). **(C)** For each condition, densitometry was used to calculate the total amount of CFTR protein, normalized to the loading control. Images were acquired using ChemiDoc XRS+ imaging system BIO-RAD and processed using Image lab 4.0 software.

As observed in **Figure 4.8 A, B**, wt-CFTR was detected in its both forms (mature and immature), however the same was not observed for F508del- and I507del-CFTR, that only appears in the immature (band B) CFTR form. For F508del-CFTR, the treatment with VX-809 promoted the appearance of mature CFTR. However, for I507del-CFTR, the treatment with the corrector did not lead to the appearance of band C. From these results we were able to: (i) determine that I507del mutation causes a processing defect in processing; (ii) VX-809 is not able to rescue the I507del processing and (iii) it is likely a class II mutation, as previously described in COS-7 and HEK293T cells [30].

Rescue of CFTR bearing this mutation will need better correctors as the defect elicited is most likely different from the one caused by F508del.

4.1.6. R553P mutation – assessment of protein processing

R553P is a missense mutation caused by a G to C transversion at cDNA position 1790 located in exon 10, causing a replacement of the amino acid residue arginine by a proline residue in position 553. Little is known about this mutation, as it is not described on *CFTR2* or at the *Cystic Fibrosis Mutation Database*, reason why we decided to study this mutation.

In order to understand how this mutation affects CFTR, HEK293T cells were transiently transfected with cDNA encoding R553P-, F508del- and wt-CFTR. Total protein was extracted and a WB was performed to assess the presence of mature and immature forms of CFTR.

Results show that the CFTR bearing R553P is detected only in its immature form (band B) similarly to F508del-CFTR (**Figure 4.9 A**). These data suggest that R553P causes a defect in CFTR processing.

We also tested if VX-809 is able to rescue R553P-CFTR. For that we used total protein extracted from HEK293T cells transiently transfected with cDNA encoding R553P-, F508del- (as a positive control) or wt-CFTR, treated with VX-809 3 μM or DMSO 0.1% v/v (as a vehicle control) for 24h and we performed a WB (**Figure 4.9**).

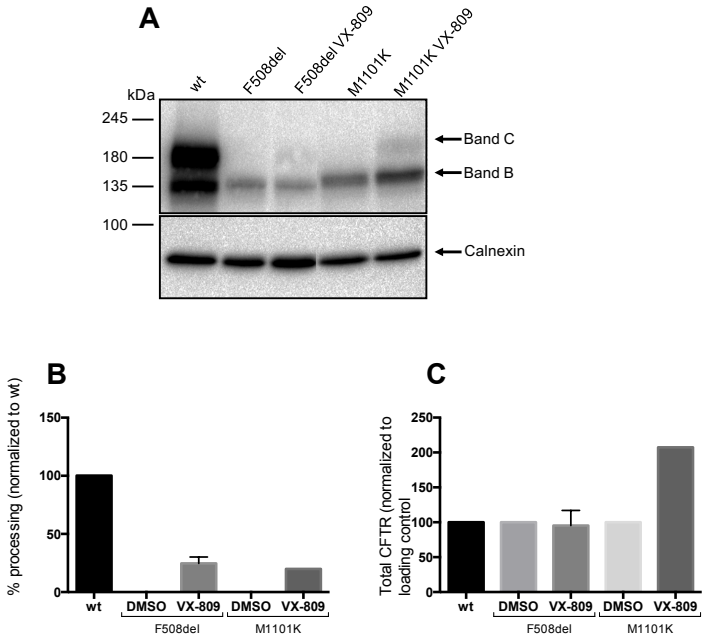


Figure 4.9. **Effect of VX-809 on processing of R553P-CFTR and F508del- (control) CFTR protein assessed by WB. (A)** Representative WB analysis of HEK 293T cells transient transfected with pcDNA5-CFTR-wt, pcDNA5-CFTR-F508del or pcDNA5-CFTR-R553P, following treatment with VX-809 (3 μM) and DMSO (0.1% v/v). **(B)** For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed. Data were normalized to the efficiency of processing of wt-CFTR (n=1). **(C)** For each condition, densitometry was used to calculate the total

amount of CFTR protein, normalized to the loading control. Images were acquired using ChemiDoc XRS+ imaging system BIO-RAD and processed using Image lab 4.0 software.

As shown in **Figure 4.9 A, B**, treatment with the corrector promoted the appearance of the mature R553P-CFTR. However, as for F508del-CFTR, levels of processing under corrector are only 13% of those for wt-CFTR processing. These results suggest that: (i) R553P has a processing defect; (ii) treatment with VX-809 is able to rescue 13% of R553P-CFTR to the cell surface and (iii) it is likely a class II mutation.

4.1.7. R560S mutation

iii) Assessment of R560S-CFTR protein processing

R560S is a missense mutation caused by an A to C transversion at cDNA position 1680 located in exon 12 leading to the exchange of an arginine residue by a serine residue at position 560 (allele frequency of 0.004%) [63], [64]. In CFTR2 [20] it is described as a CF-causing mutation when combined with another CF-causing mutation. This mutation was originally reported in an 11-year-old patient with pancreatic insufficiency, moderate lung disease and positive sweat chloride that was diagnosed at 4 months old [19].

To assess whether the R560S mutation affects CFTR processing, we produced a novel cell line expressing CFTR bearing this variant. As for the other mutations, to assess the presence of mature (band C) and immature (band B) forms of CFTR, WB was used (**Figure 4.10 A, D**). Results showed that, similarly to the F508del mutation, the protein resulting from the R560S mutation only appeared in its immature form (**Figure 4.10A, B, D, E**). This result indicates that this CFTR mutation causes a defect in CFTR processing.

Next, we tested the efficacy of small molecule compounds in rescuing R560S-CFTR as well as temperature rescue. The following compounds were tested: VX-809, VX-661 (Tezacaftor) – a second generation corrector under clinical trial, cysteamine- a US FDA-approved drug for the treatment of Cystinosis, alone and in combination with epigallocatechin gallate (EGCG) - a green tea component. The combination cysteamine+EGCG has been described to rescue F508del-CFTR [43]. Cells were incubated for 24h with: VX-809 3 μM, VX-661 5 μM, DMSO 0.1% v/v as the vehicle control, cysteamine 250 μM alone or in combination with EGCG 50 μM, and at low temperature (27^{o}C). As a positive control, compounds were also tested on F508del-CFTR in CFBE cells. Processing of R560S-CFTR was assessed by WB (**Figure 4.10 A, B, D, E**).

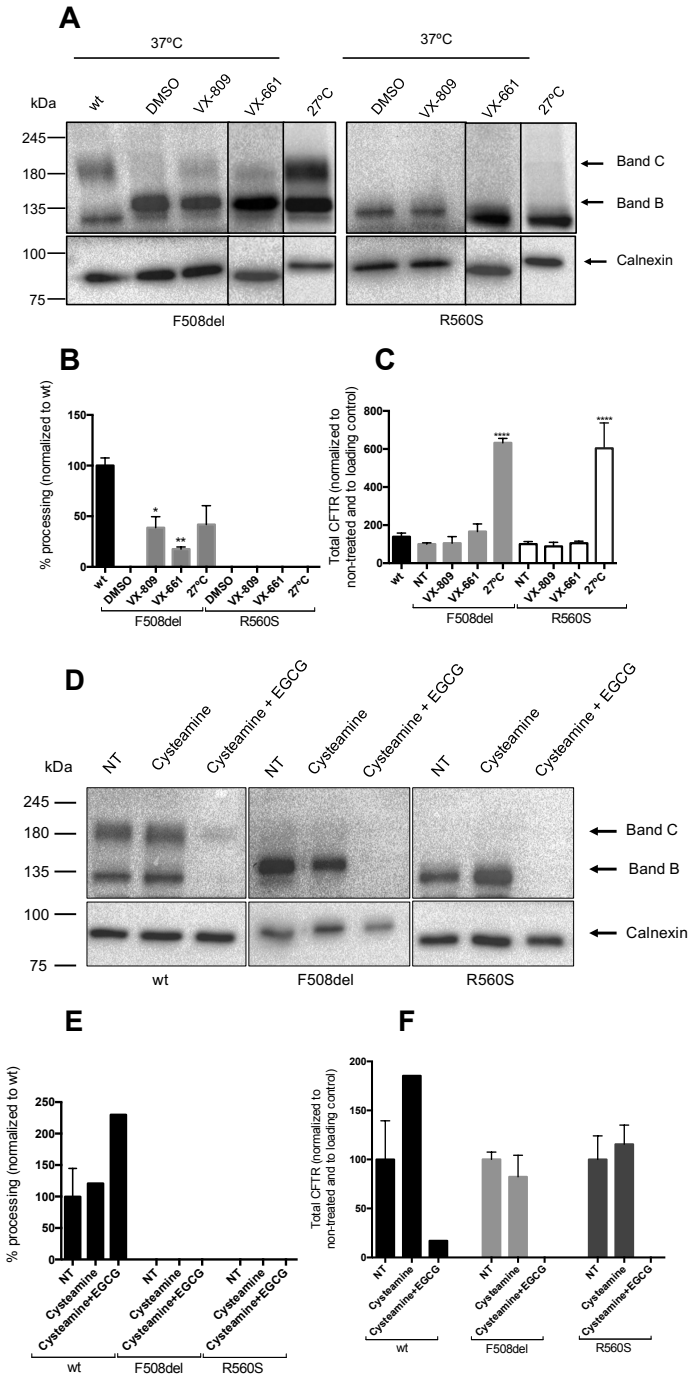


Figure 4.10. **Effect of correctors on processing of R560S-CFTR and F508del- (control) CFTR protein assessed by WB. (A)** Representative WB analysis of CFBE cells stably expressing R560S-, F508del- or wt-CFTR, following treatment with VX-809 (3 μM), VX-661(5 μM) , DMSO (0.1% v/v) or incubation at 27ºC, for 24h. **(D)** Representative WB analysis of CFBE cells stably expressing R560S-, F508del- or wt-CFTR following treatment with cysteamine (250 μM) and the combination of cysteamine and EGCG (50 μM) for 24h. **(B), (E)** For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed. Data were normalized to the efficiency of processing of wt-CFTR and are shown as mean ± SEM. **(C), (F)** For each condition, densitometry was used to calculate the total amount of CFTR normalized to the loading control and to the amount of CFTR in the non-treated sample and are shown as mean ± SEM (n=3). Images were acquired using ChemiDoc XRS+ imaging system BIO-RAD and processed using Image lab 4.0 software. * P<0.05 was considered as significant.

None of strategies used promoted the appearance of mature R560S-CFTR (**Figure 4.10**). Moreover, when the cells were treated with the combination of cysteamine and EGCG, no CFTR signal was detected (and this does not correspond to a general effect once calnexin, used as a loading control, was detected). Treatment of F508del-CFTR with either VX-809, VX-661 or incubation at low temperature promoted the appearance of mature protein. However, as for R560S-CFTR, we were not able to detect any processing for F508de-CFTR when treated with cysteamine and the combination with EGCG also lead to a total disappearance of the CFTR signal in WB.

iv) Assessment of R560S-CFTR protein localization

After assessing the processing status of the R560S-CFTR, the localization of R560S-CFTR was studied by IF. For this, we used CFBE cells stably expressing R560S-, F508del- or wt-CFTR. Cells were immunostained using two antibodies: one for CFTR and another for β-catenin to stain the plasma membrane.

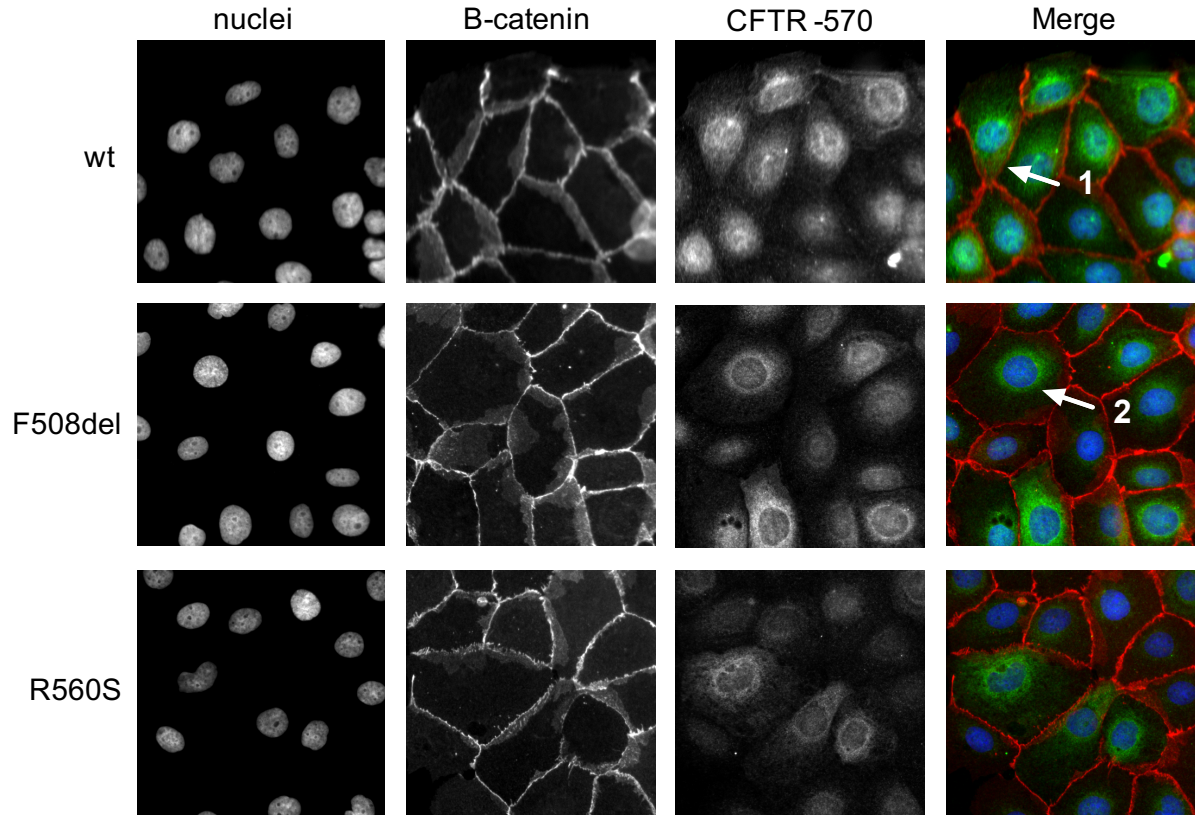


***Figure 4.11. Immunostaining images* of CFTR in CFBE cells stably expressing wt-, R560S- and F508del-CFTR. (1)** Arrow pointed to CFTR at cell surface, **(2)** Arrow pointed to intracellular CFTR. Antibodies for CFTR and β-catenin were used for cell staining. Nuclei were stained with Hoechst. (N=3)

Results from wide-field fluorescence microscopy of CFBE cells stably expressing wt-CFTR show that CFTR is present at the PM, whereas in R560s- and F508del-CFTR no protein was detected at the PM (**Figure 4.11**). Both F508del- and R560S-CFTR are located intracellularly. For R560S-CFTR, the protein seems to be mainly localized in the ER, whereas in F508del-CFTR the pattern is more diffuse evidencing some dispersion throughout the cell. These results suggest that R560S-CFTR, similarly to F508-CFTR, is retained intracellulary. The retention mechanisms might, however, be different, as suggested already by the lack of rescue with common strategies evidenced by WB.

v) Assessment of R560S-CFTR function and effect of correctors

We also assessed the functional consequences of the R560S mutation by studying the transepithelial transport in polarized monolayers of CFBE cells stably expressing this mutant by Ussing Chamber (work in collaboration N. Awatade). Cells were stimulated with forskolin 2 μM and 3-isobutyl-1-methylxantine (IBMX) 100 μM and with a CFTR potentiator (either VX-770 3 μM or Genistein 25 μM) in order to detect CFTR function. Results showed that activation of cAMP-dependent CFTR-mediated Cl^- secretion with forskolin 2 μM and IBMX 100 μM elicited a typical lumen-negative response in CFBE cells expressing wt-CFTR, that was further potentiated by treatment with genistein and inhibited with the CFTR inhibitor GlyH101 30 μM (**Figure 4.12 A**). A similar approach performed in cells expressing the R560S mutation showed no Cl^- secretion under potentiation with either VX-770 3 μM or genistein 25 μM (**Figure 4.12 B, C**). Treatment with VX-809 3 μM (in combination with potentiators) did not produce also any CFTR-mediated Cl^- secretion (**Figure 4.12 D, E**).

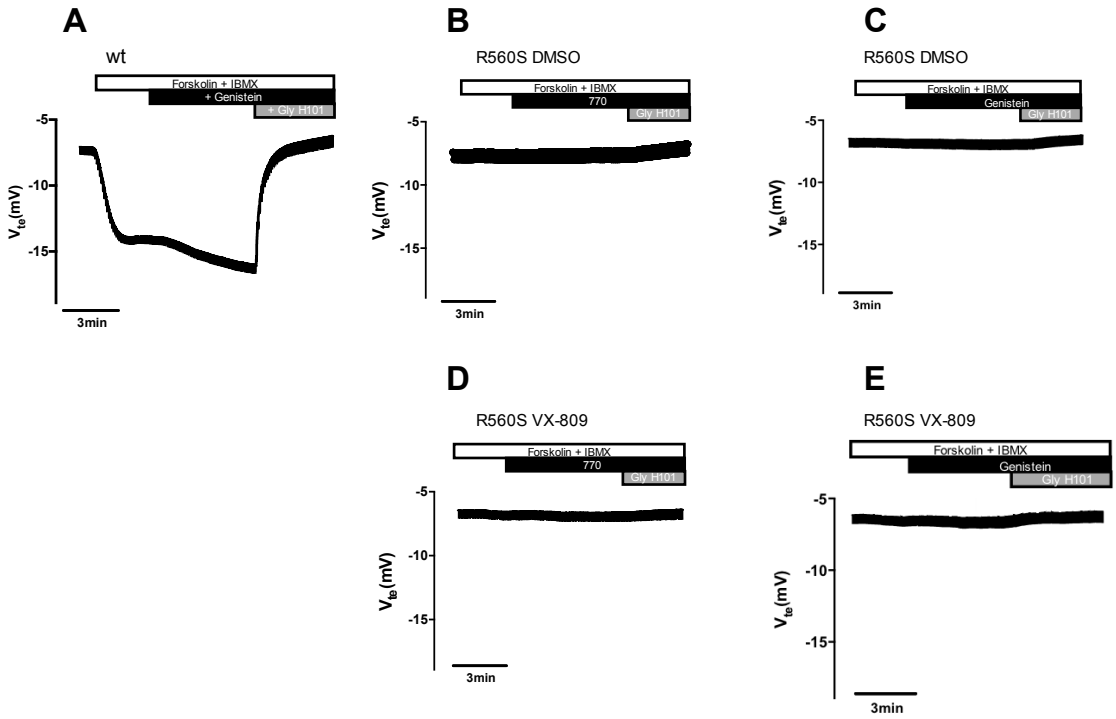


Figure 4.12. **Original Ussing Chamber (open-circuit) recordings showing transepithelial voltage measurements (V_{te}) obtained from CFBE stably expressing wt- or R560S-CFTR. (A)** CFBE stably transduced with wt-CFTR. **(B), (C), (D), (E)** CFBE cells stably transduced with R560S-CFTR. CFBE R560S-CFTR cells were pre-incubated for 24h with either 3 μM of VX-809 or DMSO (0.1% v/v) (vehicle control). A low chloride ringer solution was used at apical side to stablish a Cl^- gradient. Negative transepithelial voltage (V_{te}) deflections were observed following the application of apical forskolin (2 μM) and IBMX (100 μM) or with either VX-770 (3 μM) (B and D) or genistein (25 μM) (C and E). The latter are fully reverted by application of the specific CFTR inhibitor GylH101 (30 μM).

We also assessed the R560S-CFTR function using intestinal organoids by performing the FIS assay (work in collaboration N. Awatade). Organoid swelling under stimulation with forskolin is dependent on CFTR, thus indicative of its function. The FIS assay can also be used to assess response to CFTR modulators.

Organoids were obtained from a rectal biopsy from a CF patient homozygous for the R560S mutation (assay performed by N. Awatade). For the FIS assays, intestinal organoids were treated with

VX-809 3 µM (24h before the experiment), or in combination with VX-770 3 µM or with VX-770 alone. In this assay CFTR was stimulated with different concentrations of forskolin (**Figure 4.13**).

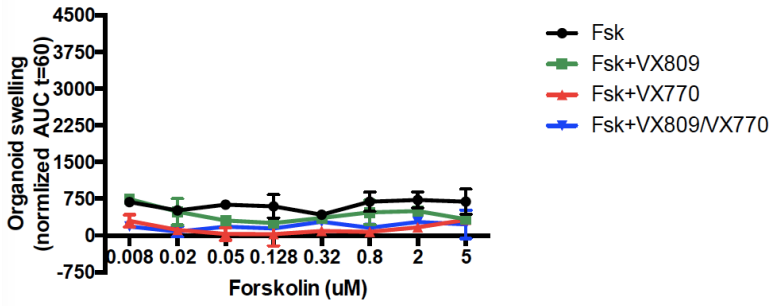


Figure 4.13. **FIS assay of organoids with the R560S mutation**. Data of FIS is expressed as the absolute area under the curve (AUC) (baseline=100%, t=60 min) of organoids incubated with VX-809 (3µM) and stimulated with Fsk (0.008 - 5µM) and VX-770 (3µM).

The area under the curve (AUC) corresponds to the area that is under the graph that represents the swelling over time. The results showed that the area did not increase to neither of the conditions studied here. This indicate that R560S organoids exhibit no function and that VX-809 did not produce significant Fsk-induced swelling compared to the non-treated control (only Fsk). The same results were obtained for VX-770 alone or in combination with VX-809.

In summary we were able to conclude that: (i) R560S mutation has a processing defect; (ii) it is likely a class II, (iii) it is not rescued by any of the strategies tested.

4.1.8. L997F mutation – assessment of protein processing

L997F is a missense substitution with a change from a leucine residue to a phenylalanine residue at position 997, resulting from a G/C transition at position 2991 in exon 17a (frequency 0.07%) [65]. Although the mutation L997F has been first described since 1992 [66], it is still highly controversial from the functional point of view. It was first reported as a polymorphism and then it was reported to cause both CFTR-related disorder (CFTR-RD) or no disease [67], [68]. According to CFTR2, this mutation does not cause CF when combined with another CF-causing variant [20]. A genetic analysis detected the R177L mutation on the same allele as L997F, revealing a complex allele (a complex allele bears two (or more) mutations in *cis*) [67], [68].

To assess the effect of L997F on CFTR processing, HEK 293T cells were transiently transfected with cDNA encoding L997F- (and wt-CFTR as control). WB was used to assess CFTR expression (**Figure 4.14 A**).

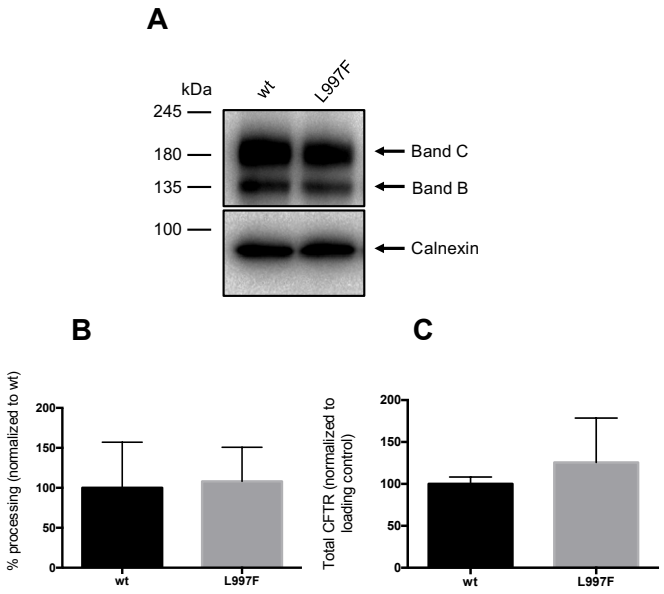


Figure 4.14. **Western Blot analysis of L997F-CFTR protein expression. (A)** Representative WB analysis of HEK 293T cells transient transfected with pcDNA5-CFTR-wt or pcDNA5-CFTR-L997F. **(B)** For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed. Data were normalized to the efficiency of wt-CFTR processing and is shown as mean ± SEM (n=2). **(C)** For each condition densitometry was used to calculate the total amount of CFTR protein, normalized to the loading control and is shown as mean ± SEM. Images were acquired using ChemiDoc XRS+ imaging system BIO-RAD and processed using Image lab 4.0 software.

As shown in **Figure 4.14 A, B**, both the immature and mature forms are detected for L997F-CFTR (similarly to wt-CFTR). These results corroborate that L997F-CFTR does not cause a defect in protein processing. Although previous publications suggest that this mutation has a functional defect. The data obtained herein does not allow a robust suggestion on the class to which this mutation belongs. Thus, functional studies are needed to confirm these results.

4.1.9.H1079P mutation – assessment of protein processing

H1079P is a missense mutation in which a histidine residue is replaced by a proline residue at position 1079, resulting from a A>C transition at position 3236 (exon 17b). This mutation was identified in two sisters with CF with age 18 and 15. The two siblings are PI and have the mutation W1282X on the other allele [19]. Little is known about this mutation and it is not described in *CFTR2*.

In order to understand how this mutation affects CFTR processing, a CFBE cell line was produced and WB used to assess CFTR expression and processing (**Figure 4.15**).

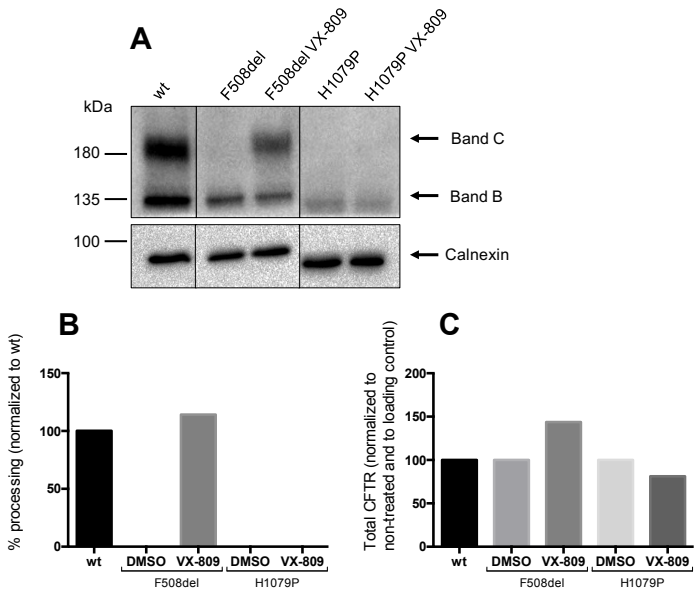


Figure 4.15. **Effect of VX-809 on processing of H1079P-CFTR and F508del- (control) CFTR protein assessed by WB. (A)** Representative WB analysis of CFBE cells stably expressing H1079P- and wt-CFTR, following treatment with VX-809 (3 μM) and DMSO (0.1% v/v). **(B)** For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed (n=1). **(C)** For each condition densitometry was used to calculate the total amount of CFTR protein, normalized to the loading control. Images were acquired using ChemiDoc XRS+ imaging system BIO-RAD and processed using Image lab 4.0 software.

CFTR bearing H1079P is detected only in the immature form (**Figure 4.15 A, B**), suggesting that the mutation causes a defect in processing.

We also tested if corrector VX-809 is able to promote the appearance of mature H1079P-CFTR. Treatment with VX-809 was not able to rescue H1079P-CFTR, as no band C could be detected (**Figure 4.15 A, B**). From these results, we conclude that: (i) H1079P mutation causes a processing defect; (ii) VX-809 is not able to rescue H1079P-CFTR and (iii) H1079P is likely a class II mutation.

4.1.10. M1101K mutation – assessment of protein processing

M1101K is caused by a T to A transversion at position 3302 leading to a change of a methionine residue to a lysine residue at position 1101. It has a frequency of 0.16% and it was first found in homozygosity in a Hutterite CF patient [47], [69]. According to *CFTR2*, patients homozygous for M1101K are pancreatic insufficient [20], [69]. Studies in cell lines suggested that this mutation is a class II mutation that causes disruption of CFTR processing and is non-functional in anion efflux assays [70]. Co-administration of C18 and C4 rescued the processing defect of M1101K-CFTR [71].

To elucidate to what extent the M1101K mutant affects CFTR expression and processing, HEK 293T cells were transiently transfected with cDNA encoding M1101K-, F508del- and wt-CFTR proteins. Results showed that the CFTR bearing M1101K is detected only in its immature form (band B). This indicates that this mutation leads to a defect in CFTR processing.

We also aimed to understand if the corrector VX-809 was able to promote the rescue of M1101K-CFTR. To this end, after transfection with pcDNA5-CFTR-wt, pcDNA5-CFTR-F508del (positive control) or pcDNA5-CFTR-M1101K, HEK 293T cells were treated for 24h with VX-809 3 μM or DMSO 0.1% v/v (vehicle control) (**Figure 4.16 A**).

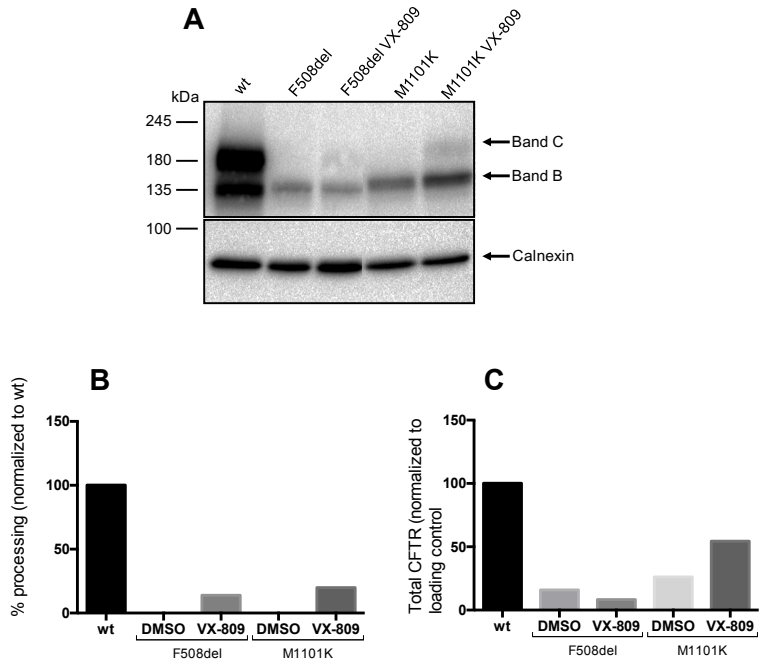


Figure 4.16. **Effect of VX-809 on processing of M1101K-CFTR and F508del- (control) CFTR protein assessed by WB. (A)** Representative WB analysis of HEK 293T cells transient transfected with pcDNA5-CFTR-wt, pcDNA5-CFTR-F508del or pcDNA5-CFTR-M1101K, following treatment with VX-809 (3 μM) and DMSO (0.1% v/v). **(B)** For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed. Data were normalized to the efficiency of processing of wt-CFTR (n=1). **(C)** For each condition, densitometry was used to calculate the total amount of CFTR protein, normalized to the loading control. Images were acquired using ChemiDoc XRS+ imaging system BIO-RAD and processed using Image lab 4.0 software.

As shown in **Figure 4.16 A, B**, treatment with VX-809 promoted the appearance of mature M1101K-CFTR (similarly to what is observed for F508del-CFTR). As for F508del-CFTR, rescue of M1101K-CFTR with VX-809 lead to an efficiency of processing that is smaller than that of wt-CFTR (20% normalized to wt-CFTR).

Results show that: (i) M1101K causes a defect in processing; (ii) treatment with VX-809 is able to rescue 20% of M1101K-CFTR and (iii) it is likely a class II mutation.

4.1.11. D1152H mutation – assessment of protein processing

D1152H (0.4% frequency)[47] results from a G to C base change at nucleotide 3454 (exon 18) resulting in an aspartic acid to histidine residues substitution at position 1152. It has been classified as a class IV mutation with residual function and abnormal chloride gating [72], [73]. *CFTR2* database describes D1152H as a mutation with variable penetrance, i.e., this variant may or may not cause CF [20]. Clinical data indicate that the phenotype of CF patients carrying D1152H are widely variable, ranging from normal to CF, usually characterized by mild pulmonary disease and PS [72], [74]. None of the D1152H subjects identified since 1999 had died or required lung transplantation and in some countries the drug VX-770/Kalydeco has already been approved for patients bearing this variant [20].

In order to understand how this mutation affects CFTR, HEK 293T cells were transiently transfected with cDNA encoding D1152H- or wt-CFTR. WB was used to assess CFTR expression (**Figure 4.17 A**).

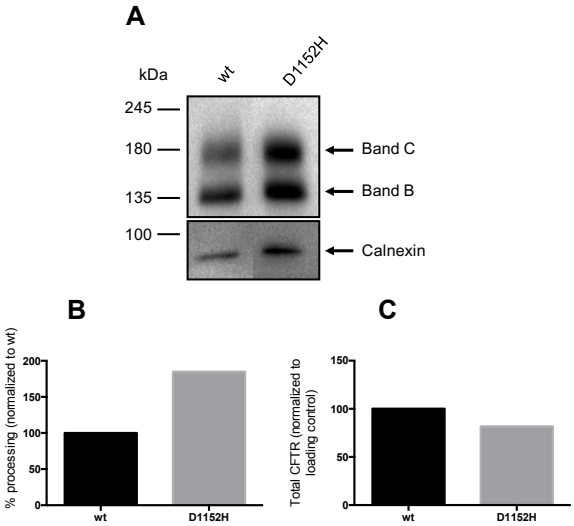


Figure 4.17. **Western Blot analysis of D1152H-CFTR protein expression. (A)** Representative WB analysis of HEK 293T cells transient transfected with pcDNA5-CFTR-wt or pcDNA5-CFTR-D1152H. **(B)** For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed (n=1).**(C)** For each condition densitometry was used to calculate the total amount of CFTR protein, normalized to the loading control. Images were acquired using ChemiDoc XRS+ imaging system BIO-RAD and processed using Image lab 4.0 software.

Similarly, to wt-CFTR, CFTR bearing D1152H was detected in both its immature (band B) and mature (band C) forms (**Figure 4.17 A, B**). From these results we were able to determine that D1152H does not affect CFTR processing and maturation, suggesting that any defect may be functional.

Based on our results, as well as on previous studies, we propose the following classification of mutations as shown in **Table 4.2**:

Table 4.2. **Summary of all the results obtained in this project.**

Mutation	Localization	Vector[a]		Stable CFBE cell line[a]	CFTR processing (band C)[b]	Rescue with VX-809[c]	Class
		Mutagenesis in pcDNA5	Cloning in pLVX-puro				
P205S	TM3	✓	✓	✓	✗	✓	IV
L206W	TM3	✓	-	-	+	✓	?
R334W	TM6	✓	✓	✓	++++	-[d]	IV
R347P	TM6	✓	-	-	++++	-	?
I507del	NBD1	✓	-	-	✗	✗	II
R553P	NBD1	✓	✓	-	✗	✓	II
R560S	NBD1	✓	✓	✓	✗	✗[e]	II
L997F	TM9	✓	✓	-	++++	-	?
H1079P	ICL4	✓	✓	✓	✗	✗	II
M1101K	TM11	✓	-	-	✗	✓	II
D1152H	Linker MSD2-NBD2	✓	-	-	++++	-	?

[a] ✓, done
[b] ✓, no band C; +, very reduced amounts of band C; ++++, processing similar to wt-CFTR
[c] ✓, rescue; ✗, no rescue; -, not done.
[d] Possible rescue with low temperature
[e] ✓, not rescued by VX-661, cysteamine, EGCG and low temperature.

5. Discussion

Herein, we studied eleven rare CFTR mutations, using novel cellular models and also organoids from a CF patient. This study focused in uncommon mutations that have not been characterized or may be misclassified. Using novel *in vitro* cellular systems (based on lentiviral transduction of parental CFBE cells) but also transient transfection of HEK cells and occasionally intestinal organoids obtained from patient-derived materials, we aimed to establish the basic defect associated with these mutations to confirm diagnosis and get information about prognosis for patients bearing these mutations. Out of the eleven studied mutations: (i) seven - P205S, L206W, I507del, R553P, R560S, H1079P and M1101K exhibit a defect in processing (lack or reduction in the amount of mature protein) and (ii) four - R334W, R347P, L997F and D1152H - exhibit both immature and mature CFTR forms suggesting that CF results from a functional defect. Among the seven for which there is a defect in processing, P205S, L206W, R553P and M1101K mutations are responsive to the corrector VX-809.

P205S is a missense mutation localized at the transmembrane domain 3 (TM3) in which a proline residue is switched by a serine residue at position 205. Patients with this mutation present a mild phenotype characterized by PS [49]. Our results in this study point to the conclusion that: (i) P205S-CFTR causes a defect in processing, since it shows only a small amount of mature CFTR but (ii) it can be rescued by the compound VX-809 and (iii) in Ussing chamber assays it showed some residual CFTR-dependent Cl^- that increased after pre-incubation with VX-809. Our results are corroborated by functional assays that also indicate that P205S still has some residual Cl^- channel function which characterizes this mutation as a mild mutation [75]. So far, there were no previous studies showing the effect of compounds on this mutation. We showed by WB (confirmed by results in our lab using the FIS assay in intestinal organoids, unpublished data by Iris S.) that VX-809 promotes the appearance of band C, with an even higher efficiency (96% of wt-CFTR processing) than for F508del (27% of wt-CFTR processing).

L206W is a missense mutation, also localized in the TM3 in which a leucine residue is substituted by a tryptophan residue at position 206. Previous reports show that CFTR with L206W has residual cAMP-dependent Cl^- currents, which is believed to be sufficient to confer a mild clinical phenotype [51], [54]. Also, immunoprecipitation studies showed that L206W produced mature protein, however the amount of band C was largely reduced in comparison with wt-CFTR [51].

Our results in this study indicate that: (i) L206W-CFTR is a mild mutation characterized by a decrease (18% of wt-CFTR processing) in band C (but not total absence) which suggests a defective processing which (ii) can be rescued by VX-809. A better characterization of this mutation in a CFBE-based cell model will allow a functional assessment.

Results obtained for L206W and for P205S, two mutations in two consecutive amino acid residues, suggest that that the middle of TM3 is a critical region for CFTR processing (**Figure 5.1**).

R334W is a missense mutation localized in the TM6 in which an arginine residue is replaced by a tryptophan residue at position 334. Clinical studies report that R334W when in homozygosity is characterized by PS but, when in heterozygosity, depending on the mutation present in the other allele can lead to PI [56], [58]. Our results point to the conclusion that: (i) R334W has a correct processing, presenting both band C and B as well as wt-CFTR which means (ii) that this mutation has probably a functional defect. Preliminary functional studies of polarized monolayers of the novel cell expressing R334W-CFTR evidence some residual function (data not shown), confirming that this mutation is probably a class IV mutation, as described previously [76]. This is also confirmed by functional studies in organoids (performed in our lab) that, besides documenting a functional defect, show that it can be rescued by treatment with the potentiator VX-770. When using the combination VX-770 and

VX-809, CFTR function is even higher. Although further functional studies are necessary, these results lead to the conclusion that R334W is probably a class IV mutation.

R347P is a missense mutation caused by a substitution of an arginine residue by a proline residue at position 347, localized on TM6. Clinical data suggests that this mutation causes PI in most of the cases, being classified as a severe mutation [61]. The results obtained in this study indicate that: (i) R347P does not has a defect in processing, since it showed both mature and immature forms of CFTR, as it is observed for wt-CFTR; so (ii) this mutation has probably a functional defect. Previous studies in FRT cells showed that R347P is correctly processed, but the magnitude of the current was reduced, classifying it as a class IV [62]. Our results showing processing for R347P-CFTR corroborate with those previous data. In order to obtain functional validation, a novel model expressing this mutant in CFBE cells is still to be needed. This would be particularly relevant as studies in rectal organoids derived from CF patients bearing R347P mutation show that this mutation is not responsive to VX-770 treatment [77]. Overall, the results obtained for this mutation are not sufficient yet to classify this mutation. Further functional studies are needed in the future.

Interestingly, both mutations studied in TM6 (**Figure 5.1**) do not lead to a defect in processing, suggesting that this segment is less critical in terms of processing – even considering that for both mutations (R334W and R347P) the aminoacid missing is the positively charged arginine and in both cases the novel aminoacid imposes, what would be expected to be, significant structural constraints – tryptophan for being an aromatic residue, with a bulky side chain, and proline, that "forces" a kink in the protein structure.

I507del mutation results from a deletion of a residue of isoleucine at position 507, localized in NDB1. Clinical studies indicate that most patients bearing this mutation are PI [47]. Our studies about this mutation suggests that: (i) It causes a defect in protein processing, with only band B being detected, and (ii) the processing defect caused by I507del was not rescued by the corrector VX-809 in HEK 293T cells, evidencing the severity of the defect, in light of this modulator. Other studies in cell lines (HEK 293T and COS-7) indicated that this mutation causes a severe defect in CFTR processing, having been classified I507del as a class II mutation. Also, the correctors C3, C4 and C18 also failed in rescue this mutation to the cell surface [30]. We still need to develop cell lines that stably express I507del-CFTR to study this mutation in terms of function but also a more generic response to rescuing strategies (low temperature, other compounds and combinations of compounds).

R553P mutation is caused by a replacement of an arginine residue by a proline residue at position 553, being localized in NDB1. This mutation is extremely rare, not being described either on *CFTR2* [20] or in *Cystic Fibrosis Mutation Database* [19]. So far, there were no previous studies regarding the molecular or functional consequences of this mutation. Our results point to the conclusion that: (i) R553P leads to a defect in CFTR processing that causes the absence of band C, and (ii) the corrector VX-809 is able to partially rescue this processing defect (13% of wt-CFTR processing). These results allow us to conclude that R553P mutation can be classified as a class II mutation, nevertheless, it is still necessary to assess its function, and for that is crucial the development of a cell line stably expressing this mutant.

R560S is a missense mutation caused by replacement of an arginine residue by a serine residue at position 560, also localized at the NDB1. This mutation is described in *CFTR2* as causing PI when combined with another mutation that also causes PI [20]. The available data reporting this mutation in FRT cells, suggests that it has a processing defect characterized by the total absence of band C [78]. Our results showed that: (i) R560S has a defect in processing that leads to the absence of band C and of membrane staining (observed by WB and immunofluorescence); (ii) the mutation is not rescued by VX-809, VX-661, cysteamine nor low temperature incubation; (iii) cysteamine and EGCG co-treatment leads to the total disappearance of CFTR (both band B and band C) and (iv) R560S did not show any residual function (assessed by Ussing chamber and FIS assays). According to our results

we propose that R560S is as a class II mutation, not rescued by available modulators. As such, there is a need to find new compounds to rescue this mutation.

Results with the three mutations located in NBD1 (I507del, R553P and R560S) confirm a critical role of this domain in the processing of CFTR (**Figure 5.1**). All 3 mutations cause defects in processing, which was rescued by VX-809 only for one of them.

L997F is a missense mutation localized at TM9 where a leucine residue is replaced by a phenylalanine residue at position 997. According to *CFTR2*, this mutation does not cause CF when combined with another CF-causing variant [20]. Our results suggest that L997F does not affect CFTR processing, as both forms of CFTR are detected similarly to wt-CFTR. According to previous report, this variant was believed to be a polymorphism or to cause either CFTR-RD or no disease at all, being highly controversial from the functional point of view [67], [68]. This information corroborates our results that L997F does not have a processing defect, so any possible defect would be functional, thus needing more detailed characterization based on a novel cell line expressing CFTR bearing this variant. Data in the literature indicate that the mean [Cl^-] conductance of L997F-CFTR (as % wt-CFTR) is of 22% [79], thus confirming the *CFTR2* non-CF-causing classification. However, L997F was also described in a 10-year old individual with the diagnosis of atypical CF with recurrent pancreatitis, sinusitis with nasal polyps, and mild lung disease [80] Moreover, it was described that the combination of L997F and R117L on the same allele causes a more severe phenotype than L997F alone [67]. It is thus possible that L997F itself is not a CF-causing mutation, only when *in cis* with R117L (or another variant).

H1079P is a missense mutation localized in intracellular loop 4 (ICL4) in which a histidine residue is substituted by a proline residue at position 1079 [19]. There is almost no information about this mutation that is extremely rare and is not included in *CFTR2*. Our results point to the conclusion that: (i) H1079P causes a severe defect in processing and (ii) it is not rescued by the corrector VX-809. Further functional studies are needed using the novel cell line stably expressing H1079P-CFTR. Studies performed in our lab using rectal organoids from a CF-patient heterozygous for this mutation corroborate our results that the H1079P mutation does not respond to VX-809. In summary, our results in combination with the functional results in our lab point to the conclusion that H1079P is a class II mutation.

Results obtained with H1079P mutation located in ICL4, confirm the relevance of this segment for CFTR processing. The segment mediates relevant interdomain interactions with NBD1.

M1101K is a missense mutation, is localized in transmembrane domain 11 (TM11), in which a methionine residue is replaced by a lysine residue at position 1101. It is described in *CFTR2* as causing PI when combined with another mutation that also causes PI [20]. Results obtained in this study revealed that: (i) M1101K leads to a defect in protein processing and (ii) treatment with VX-809 was able to rescue some of the protein to the cell surface (20% of wt-CFTR processing). Previous studies in cell lines suggested that M1101K is a class II mutation that causes a defect in CFTR processing [71], which corroborate our results also in cell lines. Additionally, it was observed through anion efflux assays that M1101K-CFTR was non-functional [70]. Additional functional studies are needed in the future to confirm these results. Our results showing that VX-809 is able to partially correct the processing defect caused by M1101K are in agreement with previous reports that correctors C18 and C4 were able to rescue the trafficking of M1101K-CFTR to the cell surface [71].

D1152H is a missense mutation caused by a switch of an aspartic acid residue to a histidine residue at position 1152, localized on the linker between the TM12 and NDB2. Phenotypes of CF patients carrying this mutation have been described as widely variable [73], [74], but usually characterized by mild pulmonary disease and PS [72]. Our results indicate that D1152H does not cause a defect in processing, since both forms of CFTR were detected. Previous studies have described that this mutation has residual function and abnormal chloride gating, classifying it as a class IV

mutation. Our results support the observation that this mutation does not have a defect in processing. However, further functional studies are required to evaluate the effect of D1152H mutation in CFTR function and to be able to confirm its classification as a class IV mutation.

In summary, here we studied eleven rare CFTR mutations. Four of them were studied using novel CFBE-based cell models, namely, P205S, R334W, R560S and H1079P. We classified three mutations that have not been classified so far R553P, R560S and H1079P - two of them are not even described in *CFTR2* - and we also confirmed the already described classification for the remaining mutations, P205S, L206W, R334W, I507del and M1101K. Furthermore, we were able to show that VX-809, already approved for F508del – in combination with VX-770 -, can also promote the rescue of four of the mutations studied - P205S, L206W, R553P and M1101K.

The cell lines developed here are extremely important to study mutations affecting very few patients worldwide and for which outcome prediction is difficult. Moreover, by testing the response to approved drugs in these systems, we can predict the response of patients carrying these mutations to such drugs. Although patient derived materials are a more physiological relevant model, most mutations are found in heterozygosity, being difficult to discern if an observed effect comes from one or other allele. Therefore, it is essential the production and use of these cell lines in the study of rare mutations. From all CF patients, few are the ones who carry a "drug-approved" mutation and thus, it is not known whether they can benefit from CFTR potentiator/corrector therapy. Elucidation of the molecular and cellular effects of these mutations is useful to predict disease severity and to provide the scientific basis for the development of targeted compounds for mutation-specific and/or personalized therapy, as we did in this work.

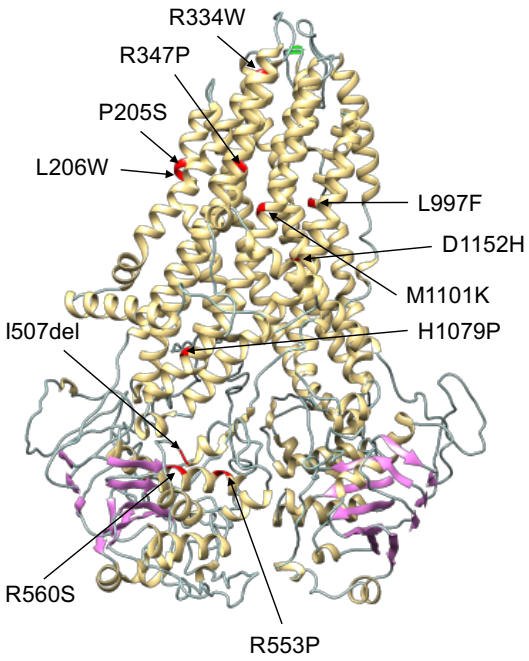


Figure 5.1. **Representation of the location of each mutation in study on the CFTR structure.** The MSDs are represented in yellow, the NBDs are represented in pink, and each mutation are represented in red. The PDB file was obtained from I-TESSER server [81]–[83].

6. Future Perspectives

The results obtained here with the cellular models generated are very encouraging to further pursue the study of "orphan" CFTR mutations, as well as to repurpose the existing therapies used to common mutations in these rare mutations. Since insertion of the mutations, cloning and development of stable cell lines of a high number of mutations were a limiting and an on-going step for this work, additional experiments can now be planned and design for future work, as will be described next.

Firstly, it is necessary to clone into pLVX-puro vector the remaining mutations that were inserted into pcDNA5 by site-directed mutagenesis, namely: L206W, R347P, I507del, R553P, L997F, M1101K and D1152H. This will allow us to perform functional studies, such as assessment of transepithelial transport in Ussing chambers, in order to better characterize these mutations.

Secondly, since the cell lines here developed seem to have reduced CFTR expression compared to other models (e.g. equivalent CFBE cell lines expressing wt-CFTR and produced previously in other labs [84]), it would be extremely important to find a way to increase this expression. To this end, we could try to find a way to do sorting of the cells (a difficult task since we are overexpressing a non-tagged version of CFTR) or do another transfection of the already transfected cells.

Thirdly, it would be interesting to assess the cell localization of each mutant, by immunofluorescence assays, to confirm the results obtained here by Western Blot.

Fourthly, as previously mentioned, it is necessary to perform functional studies of the mutations that are correctly processed, and thus probably have a functional defect, namely: R334W, R347P, L997F and D1152H. Moreover, it would be also interesting to study by functional assays the mutations that we showed here that are rescued by VX-809.

Fifthly, for the mutations that were not rescued or only were not completely rescued by VX-809 (I507del and H1079P) it would be important to test other compounds, as for instance, VX-661, C4, C3, C18, [41], [85]–[87] or even the combination of the different compounds in order to understand if they could restore (individually, additively or synergistically) the processing of these CFTR variants. Furthermore, not only but particularly for the mutations that appear to be unrescuable with available modulators, it is important to understand their trafficking/degradation pathways by exposing each of the CFTR mutants to modulators (e.g. proteasome or lysosome) to evaluate the effect of different processes in the misprocessing and putative rescue of CFTR bearing such variants.

Finally, although here we only used cell models to study these mutations, it would be interesting to confirm the results obtained in cell lines. Patient materials, such organoids or primary cells, could be used to test the drugs that here we show that lead to CFTR rescue. This is however an approach that is being followed in our lab.

The continuation of the work developed during this project is thus crucial to deepen the knowledge of each of these CFTR orphan mutations. The understanding of the effect of each mutation and the compounds that can lead to its rescue would be of high importance to all the patients carrying these mutations and for whom there are no approved therapies.

7. References

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